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# STUDIES ON THE DISTRIBUTION, PROPERTIES, AND ISOLATION OF A NATURALLY OCCURRING PRECURSOR OF NICOTINIC ACID\*

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Analysis of wheat bran and certain other natural materials for nicotinic acid has revealed the presence therein of a water-soluble substance which appears to be an unidentified derivative or precursor of this vitamin (1, 2). This substance, which will be called the "precursor," is characterized by extreme lability toward alkali. In fact, its existence was first recognized as a result of an increase in the apparent nicotinic acid content of wheat products following alkali treatment. Such observations have naturally raised a question as to whether the increase is actually caused by liberation of additional nicotinic acid, rather than by some other substance which interferes with the analytical method used, and, if so, whether the precursor is biologically active *per se*.

In this and the following paper evidence is presented which indicates that the precursor quite definitely is a derivative of nicotinic acid, that it is not identical with any of several known compounds which yield the vitamin on hydrolysis, and that it probably possesses nicotinic acid activity toward higher animals. The present paper contains an account of the chemical study of the substance, and of attempts to separate it from free nicotinic acid.

## *Experimental Results*

Nicotinic acid activity was followed by the microbiological method (3, 4). Values given for the amount of precursor in any sample are expressed in terms of nicotinic acid, and represent the differences found before and after alkali treatment. This treatment consisted in exposing the material to the action of 0.25 to 0.73 per cent sodium hydroxide at room temperature for 5 minutes.

Since the increase in apparent nicotinic acid after alkali treatment might have been caused by stimulants of a fatty nature, two typical samples (aqueous extracts of whole wheat and of wheat bran) were filtered at pH 4.5, and the filtrates extracted three times with ether. The samples

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prepared in this manner showed the same precursor content as those assayed directly. This result is in agreement with previous observations on the relative insensitivity of *Lactobacillus arabinosus* to fatty acid stimulation (4).

*Extraction Studies and Distribution of Precursor in Biological Materials*—Assay results on a number of samples are collected in Table I. These samples were autoclaved with 100 parts of water for 15 minutes at 15 pounds per sq. in. pressure. Solid residues were filtered off, washed twice with water, and the filtrates assayed. The amounts of precursor given in Table I should be regarded as minimum values, since the extraction

TABLE I  
*Distribution of Precursor in Natural Materials*

Sample	Nicotinic acid		Precursor  γ per gm
	Before NaOH treatment	After NaOH treatment	
	γ per gm	γ per gm	
Wheat milling fractions			
Whole wheat	42	62	20
Bran	204	250	46
Shorts	104	129	25
Red dog	60	80	20
Germ	53	53	0
1st clear flour	16	23	7
2nd " "	35	41	6
Vitab, Lot 1*	1530	1950	420
" " 2*	1360	1770	410
Dehydrated white potato	26	51	25

\* Average of five assays

was probably not complete and there is a possibility that some hydrolysis of the precursor occurred during the extraction.

In order to secure additional information on these points both the stability of the precursor to hydrolysis and the efficiency of various extraction methods were further studied. The hot water extracts shown in Table II were made by boiling the material for 60 to 80 minutes with 20 parts of water by weight. The cold water extract was made by homogenizing 50 gm. of the bran with 500 cc. of water in a Waring blender and allowing the mixture to stand overnight at room temperature. The solid matter was filtered off and extracted once more in the same manner. The results given in Table II show that the precursor is completely hydrolyzed at pH 10 but only slightly at pH 8 when the solutions are held at 37°. Autoclaving at 62, the natural pH of the wheat bran water ex-

tract, or heating for 1 hour at 100° also brought about a small amount of hydrolysis

The extractions summarized in Table III were carried out by stirring, refluxing, or autoclaving a mixture of 10 gm of the sample with 100 cc of the solvent, except in the last three cases in which 0.5 gm portions of the bran were used with 100 cc of the solvent. It will be noted that

TABLE II  
*Stability of Precursor to Hydrolysis*

Material						Conditions of treatment	Nicotinic acid found <i>γ per gm*</i>
Hot	water	extract	of	whole	wheat	No treatment	23.6
"	"	"	"	"	"	2 N HCl, † 60 min, 100°	40.0
"	"	"	"	"	"	0.1 N NaOH, 60 min, 100°	43.1
"	"	"	"	"	"	0.25% NaOH, 5 min, 25°	41.9
Cold	"	"	"	wheat	bran ‡	No treatment	114
"	"	"	"	"	"	pH 6.2, 60 min, 100°	123
"	"	"	"	"	"	" 6.2, 15 " 120°	131
"	"	"	"	"	"	" 6.2, 60 " 120°	137
"	"	"	"	"	"	" 7.4, 3 hrs, 37°	112
"	"	"	"	"	"	" 7.4, 6 " 37°	117
"	"	"	"	"	"	" 8.0, 3 " 37°	122
"	"	"	"	"	"	" 8.0, 6 " 37°	124
"	"	"	"	"	"	" 10.0, 3 " 37°	171
"	"	"	"	"	"	" 10.0, 6 " 37°	174
"	"	"	"	"	"	0.3 N NaOH, 5 min, 25°	171
Hot	"	"	"	"	" †	No treatment	198
"	"	"	"	"	"	pH 7.4, 3 hrs, 37°	183
"	"	"	"	"	"	" 8.0, 3 " 37°	207
"	"	"	"	"	"	" 10.0, 3 " 37°	264
"	"	"	"	"	"	0.3 N NaOH, 5 min, 25°	252

\* Based on the original whole wheat or wheat bran

† Sufficient reagent was added to the original extract to bring the solution to the indicated concentration

‡ The same sample of wheat bran was used in each of these experiments

§ Buffered with Clark and Lubs buffer mixtures, and the pH values checked on the glass electrode to within  $\pm 0.1$  unit

the highest ratio of precursor to free nicotinic acid was found in the 95 per cent alcohol extract, but that a larger total amount of precursor was removed with 0.1 N hydrochloric acid at 100°. This result, incidentally, demonstrates that the precursor is not extensively hydrolyzed under these conditions, although it is destroyed by N sulfuric acid in the autoclave (5) or by 2 N hydrochloric acid at 100° (Table II). Furthermore, the total



nicotinic acid activity taken out by 0.1 N hydrochloric acid is much less than the maximum removed by N sodium hydroxide or N sulfuric acid

Attempts were made to extract the precursor with several organic solvents other than alcohol. 1 gm samples of vitab, a rice bran concentrate (Lot 2, Table I), were twice extracted with 50 cc portions of the solvent by thorough shaking in a separatory funnel. The combined ex-

TABLE III  
*Extraction of Nicotinic Acid and Precursor from Wheat Bran*

Solvent	Conditions		Nicotinic acid in extract	Precursor in extract	Ratio of precursor to nicotinic acid
	Time	Temperature			
	min	C	$\gamma$ per gm *	$\gamma$ per gm *	
"	60	Room	106	52	0.49
"	60	100	116	46	0.40
0.1 N HCl	60	Room	114	71	0.62
0.1 " "	60	100	124	86	0.69
50% ethanol	60	Reflux	120	47	0.39
95% "	60	"	57	52	0.91
Water	30	120	230		
N NaOH	30	120	280	0	
" H <sub>2</sub> SO <sub>4</sub>	30	120	277	0	

\* Expressed on the basis of the original wheat bran

TABLE IV  
*Solvent Extractions of Vitab*

Solvent	Nicotinic acid in extract	Precursor in extract
	$\gamma$ per gm *	$\gamma$ per gm *
Acetone	860	0
Toluene	36	14
Skellysolve B	<3	<7
Carbon tetrachloride	<10	0
Ethyl acetate	568	116
" ether	0	0

\* Expressed on the basis of the original vitab

tracts were taken to dryness from a 60° water bath at diminished pressure, and the residues taken up in water for assay. The results given in Table IV show that none of the solvents removed an appreciable amount of the precursor with the exception of ethyl acetate, which extracted about one-fourth of that originally present in the vitab. Even this solvent, however, offers little advantage for isolation work since it also extracts nicotinic acid.

Although the precursor is obviously not freely soluble in non-polar solvents, it seemed desirable to try exhaustive ether extraction. Accordingly, a water extract of whole wheat was adjusted to pH 7, and subjected to continuous extraction with ether in a liquid-liquid extractor for 6 hours. The aqueous phase was found to contain 24.7 and 15.4  $\gamma$  of nicotinic acid and precursor respectively per gm of the original wheat, whereas the corresponding values before ether extraction were 23.6 and 18.3  $\gamma$ . Another portion of the wheat extract was first treated with alkali in the usual way, and then subjected to continuous ether extraction. The aqueous phase before and after extraction contained 41.9 and 37.5  $\gamma$  of nicotinic acid per gm of wheat respectively. The observed differences are only slightly larger than the error of the microbiological assay method, and are probably not significant. At any rate, both the precursor and the bulk of the active material liberated by alkali act in the same manner toward ether extraction as does free nicotinic acid.

*Precipitation Studies*—Several extracts containing the precursor and nicotinic acid were treated with common precipitating agents. In the case of lead, mercury, and silver precipitations, the excess metallic ions were removed from the filtrates as the insoluble sulfides and the precipitates were also decomposed with hydrogen sulfide. The sulfides were filtered off, washed with water, and the filtrates assayed after removal of dissolved hydrogen sulfide. The results given in Table V show that in several cases a large portion of the precursor could not be accounted for in either the filtrate or precipitate. The discrepancy is probably due to adsorption on the metallic sulfides, but no effort was made to verify this hypothesis. In general this type of procedure does not appear suitable for concentrating the precursor, or separating it from nicotinic acid.

*Adsorption and Elution*—The results of some preliminary attempts to remove either the precursor or nicotinic acid from aqueous solution by selective adsorption are collected in Table VI. The materials were dissolved in sufficient water to make an approximately 1 per cent solution, the pH adjusted, and the adsorptions carried out by adding an amount of adsorbent roughly equal to the amount of solids in the solution and stirring mechanically for 1 hour at room temperature. In most cases both substances were adsorbed about equally well.

In further experiments it was found that the precursor was not adsorbed by Darco G-60 from acidic alcohol solutions, under the conditions described by Melnick and Field (6) for decolorizing extracts before chemical determination of nicotinic acid.

Attempts to elute non-adsorbates soon showed that the precursor was held much more tightly than the nicotinic acid. It was found that pure nicotinic acid was quantitatively adsorbed by Darco G-60 from

aqueous solution at pH 4, and was quantitatively removed by elution with a 3:2 mixture of pyridine and methyl alcohol. Furthermore, it was possible to determine the nicotinic acid microbiologically while it was still adsorbed on the charcoal, simply by adding a suspension of the

TABLE V  
*Behavior of Precursor toward Precipitants\**

Preparation used	Reagent	Nicotinic acid in		Precursor in	
		Filtrate	Ppt	Filtrate	Ppt
Vitab dissolved in water	Basic lead acetate	27	19	13	39
" " " "	Phosphotungstic acid	24		15	
" " " "	Picric acid	93		117	
" " " 95% ethanol	Saturated alcoholic HgCl <sub>2</sub>	26	18	17	11
" " " 50% "	Basic lead acetate	42	15	37	16
" " " water	AgNO <sub>3</sub>		5		18
Filtrate from AgNO <sub>3</sub> pptn of vitab	Basic lead acetate	47	19	48	28
Filtrate from 95% ethanol pptn of wheat bran extract	" " "	67	17	16	5

\* The figures indicate percentages of the original totals

TABLE VI  
*Adsorption of Nicotinic Acid and Precursor*

Preparation used	Ad-sorbent	pH of solution	Nico-tinic acid in filtrate	Pre-cursor in filtrate
			per cent of original	per cent of original
Vitab	Norit A	4	5.6	1.4
"	Inulin	4	87	104
"	Amberlite 1R-1*	4	95	65
"	Unactivated wood charcoal	4	82	104
Alcohol extract of wheat bran	" " "	4.5	77	52
" " " " "	" " "	7.0	77	54
" " " " "	" " "	1.0	87	53

\* Obtained from the Resinous Products and Chemical Company, Philadelphia

adsorbate directly to the assay tubes. Adsorbates carrying both the precursor and nicotinic acid, after having had the latter removed by pyridine-methanol elution, showed little or no activity when assayed directly, but yielded additional nicotinic acid when treated with dilute

sodium hydroxide or with alcoholic potassium hydroxide. It was considered that this additional activity represented the precursor content of the adsorbates. These procedures thus made it possible to determine both the precursor and the free acid directly on the adsorbates, as well as in the various eluates and filtrates.

When these methods were applied to a quantitative study of various adsorbates, the surprising observation was made that they sometimes contained considerably more precursor than the starting materials, although the over-all balance of total nicotinic acid activity was nearly perfect. For example, the results shown in Table VII were obtained when an aqueous solution of 10 gm of vitab was adsorbed with norit A, and the charcoal eluted twice with pyridine-methanol.

TABLE VII

*Fractions Obtained on Adsorption with Norit A and Elution with Pyridine Methanol*

Starting material (10 gm of vitab), nicotinic acid 15.29 mg, precursor 4.19 mg, total activity 19.48 mg

	Nicotinic acid	Precursor
	mg	mg
Filtrate from adsorption	0.85	0.06
1st eluate	8.20	0.60
2nd "	4.00	0.0
Charcoal adsorbate	1.00	4.97
Total	14.05	5.63
Total precursor recovered, %	134	
" activity " %	101	

A similar result was obtained when a hydrochloric acid extract of wheat bran was precipitated with 75 and 95 per cent concentrations of ethyl alcohol (Table VIII).

It is probable that the high recoveries of the precursor are attributable to a partial utilization of the compound by the bacteria when it is in solution but not when it is adsorbed on charcoal.

In the light of the above experience a charcoal adsorbate was prepared in such a manner as to contain a maximum amount of precursor and a minimum quantity of free nicotinic acid. 2 kilos of wheat bran were extracted by stirring with 10 liters of 0.1 N hydrochloric acid at 100° for 1 hour. The mixture was filtered and the insoluble portion reextracted twice in the same manner. The combined extract was adjusted to pH 4.0 with sodium acetate, and stirred for 1 hour at room temperature with 150 gm of Darco G-60. The charcoal was filtered off, washed, dried,

eluted with pyridine-methanol, and dried again. The product was found to contain a total of 25.4 mg of nicotinic acid and 343 mg of precursor (0.12 and 1.65 mg respectively per gm of dry eluted adsorbate). This corresponds to 171  $\gamma$  of precursor per gm of the original wheat bran, a much higher value than any previously noted (Table III). The above adsorbate also represents the maximum separation of nicotinic acid and precursor which has so far been achieved, since it contains 13.5 times as much precursor as nicotinic acid. This preparation was designated Charcoal Adsorbate A.

A similar preparation was made by adsorbing vitab with norit A, and eluting with pyridine-methanol. Many efforts were made to elute the precursor from these adsorbates without hydrolyzing it, but the results, which are collected in Table IX, were not encouraging. The procedure

TABLE VIII

*Fractions Obtained on Precipitation with Ethyl Alcohol*

Starting material (extract from 50 gm of wheat bran), nicotinic acid 9.00 mg, precursor 3.15 mg, total activity 12.15 mg

	Nicotinic acid	Precursor
	mg	mg
75% alcohol ppt	0.30	0.12
95% " "	4.80	3.40
95% " filtrate	1.88	1.45
Total	6.98	4.97
Total precursor recovered, %	158	
" activity "	98	

used in these elution experiments was to stir 1 gm of the adsorbate with 100 cc of the eluant for 1 hour at 50-70°.

*Dialysis*—Following the suggestion of Andrews *et al* (2), we attempted to separate the precursor from nicotinic acid by dialysis. An aqueous solution of vitab was placed in a cellophane sac and dialyzed against running tap water for 24 hours at pH 7. The liquid remaining in the sac then contained nicotinic acid and precursor equivalent to 393 and 202  $\gamma$ , respectively, per gm of the original vitab. Since the corresponding figures before the dialysis were 1360 and 410  $\gamma$  per gm, it is apparent that the free acid dialyzes through cellophane more rapidly than does the precursor.

In the hope of effecting a better separation, electrodialysis was also tried. A wheat bran extract containing 3240 and 1725  $\gamma$  of nicotinic acid

and precursor, respectively, in a volume of 5 cc was placed in Cell 5 of an electrodialysis apparatus consisting of 11 cells separated by cellophane membranes. 5 cc of water were placed in each of the other cells, and a current of 4 milliamperes was passed for 24 hours. The results are given in Table X. Both substances moved toward Cell 11, the cathode, but the bulk of each remained in Cells 6, 7, and 8. The pH of these cells at the end of the experiment varied from 3.08 to 3.48. A determina-

TABLE IX  
*Elution Studies on Charcoal Adsorbates*

Preparation eluted	Eluant	Nicotinic acid in eluate†	Precursor in eluate†
		$\gamma$ per gm	$\gamma$ per gm
Norite A adsorbate of vitab (370 $\gamma$ precursor and 35 $\gamma$ nicotinic acid per gm dry adsorbate)	50% ethanol	0	0
	95% "	0	0
	Acetone	0	0
	Ethyl acetate	57	27
	0.05% HCl in ethanol	50	66
	0.1 N HCl	0	0
	Ca 1% $\text{NH}_3$ in ethanol	217	2
	" 1% " " acetone	212	20
	4% acetic acid	0	0
Darco G 60 adsorbate of wheat bran extract (1650 $\gamma$ precursor and 120 $\gamma$ nicotinic acid per gm dry adsorbate)	1% ethyl alcoholic KOH	1750	3
	0.25% $\text{NH}_3$ in ethanol	0	0
	0.1% quinine sulfate	0	0
	30% KCl	0	0
	4% acetic acid	0	0
	0.1 M phosphate buffer, pH 8.0	0	0
	10% urea	0	0
	2% glycine	0	0
	Saturated $\text{KHCO}_3$	0	0
	0.05% ethyl alcoholic KOH	682	88

\* Both preparations had been eluted with pyridine-methanol and dried before the work summarized in this table was carried out.

† Expressed per gm of dry adsorbate.

tion of the isoelectric point of pure nicotinic acid gave a value of 3.47. Apparently the precursor also is a zwitter ion, with an isoelectric point in the same region. It should be noted, however, that approximately two-thirds of the precursor was hydrolyzed during the electrodialysis.

*Cyanogen Bromide Reaction*—A sample of vitab was treated with cyanogen bromide under the experimental conditions described by Swaminathan (7) for the quantitative determination of nicotinic acid. The procedure involved reaction with cyanogen bromide at 70° for 10 minutes.

followed by treatment with aniline. The mixture was then concentrated to a small volume *in vacuo* and assayed. 51 per cent of the nicotinic acid and 2.5 per cent of the precursor were still intact. A control run with the reagents alone showed no effect on the bacterial assay.

Since so much of the nicotinic acid survived the treatment with cyanogen bromide, the reaction was tried with pure nicotinic acid under a variety of experimental conditions. In 5 minutes at room temperature about half the acid was destroyed, while at 70° for 1 hour the destruction ranged from 75 to 90 per cent. Lower temperatures and shorter times gave intermediate results.

TABLE X

*Electrodialysis of Wheat Bran Extract*

The sample contained 3240  $\gamma$  of nicotinic acid and 1725  $\gamma$  of precursor before dialysis.

Cell No	pH	Nicotinic acid	Precursor
		$\gamma$	$\gamma$
1	1.72	0	0
2	1.93	0	0
3	2.60	0	0
4	2.78	0	0
5	2.88	90	15
6	3.08	1100	75
7	3.28	1400	315
8	3.48	1550	115
9	5.85	150	40
10	11.98	0	0
11	12.2	0	0
Total		4290	560

## DISCUSSION

The evidence for the existence of the precursor and all the information available regarding its occurrence and properties are based on the results of microbiological assay before and after alkali treatment. The interpretation of these results rests on the assumptions that *Lactobacillus arabinosus* is completely unable to utilize the intact precursor, and that no substance other than nicotinic acid capable of influencing the bacterial growth is produced by the action of the alkali.

No data are at hand which cast doubt on the validity of the latter postulate, but the former seems questionable. The presence of much more than the expected amount of precursor in several charcoal adsorbates suggests that the organism can partially utilize the precursor in aqueous

solution, but not when it is adsorbed on charcoal. If this is actually the case, the values given for free nicotinic acid (before alkali treatment) are too high, and the precursor values correspondingly low. Likewise, on this basis, the failure to achieve a complete separation of the two factors would be expected, and it may be, therefore, that the properties of the two are not so closely similar as would appear from the studies carried out to date.

It has been reported (5) that the precursor is hydrolyzed by incubation at 37° both at pH 8.0 and 1.0. The results obtained in the present work do not confirm this report, and in fact indicate a considerably greater stability in acid solution. However, the precursor was found to be hydrolyzed at pH 10 *in vitro*, and it is quite possible that it is broken down under the conditions in the intestinal tract of higher animals. It has also been found that the precursor is not hydrolyzed by taka-diastase and papain (5).

Although little progress toward isolation of the precursor has been made, the work which has been reported together with newer biological studies (8) shows quite definitely that it is a complex molecule readily hydrolyzable to form nicotinic acid. It does not appear to be a quaternary pyridinium base of the type of coenzymes I and II, because it is destroyed even more rapidly by cyanogen bromide than is nicotinic acid itself. Likewise it is probably not a simple ester of nicotinic acid, although such esters are very labile to alkali, because of its insolubility in non-polar solvents and zwitter ion behavior on electrodialysis.

It is more likely that the precursor is a derivative of nicotinic acid consisting of the nicotinyl radical attached to a substituent bearing functional groups which render the entire molecule acidic and water-soluble. The available data do not warrant further speculation as to the nature of this substituent or the manner of its linkage with the nicotinic acid moiety.

#### SUMMARY

The substance in extracts of wheat bran and certain other biological materials which on mild alkali treatment becomes capable of stimulating the growth of *Lactobacillus arabinosus* is very probably a nicotinic acid derivative. This "precursor" was not readily separated from free nicotinic acid, as judged by microbiological assay, by various extraction, precipitation, adsorption, elution, or dialysis procedures. A charcoal adsorbate, carrying 13.5 times as much precursor as nicotinic acid, was prepared, but the precursor could not be eluted from it.

Evidence is presented which suggests that the precursor is a derivative of nicotinic acid consisting of the nicotinyl radical attached to a substituent bearing functional groups which render the entire molecule acidic



and water-soluble. Methods of extracting and estimating the amount of the precursor in various materials, as well as its stability under different conditions, also were studied.

The authors wish to express their appreciation to Dr J W Porter who assisted in carrying out the precipitation experiments described in this paper.

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# THE BIOLOGICAL ACTIVITY OF A PRECURSOR OF NICOTINIC ACID IN CEREAL PRODUCTS\*

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As a result of difficulties experienced in attempting to separate nicotinic acid from an alkali-labile "precursor" of this vitamin found in certain food products (1, 2) no conclusive evidence has so far been brought forward to show whether the precursor itself possesses biological activity as a source of nicotinic acid for higher animals. In the present work a number of known compounds related to nicotinic acid, as well as several preparations of the precursor, have been assayed by the bacterial method and with dogs and chicks in the hope of throwing some light on this question.

## EXPERIMENTAL

*Compounds and Preparations*—Ethyl, propyl, and butyl nicotates were prepared by reaction of the appropriate alcohol with nicotiny chloride (3), and were redistilled at reduced pressure before use. Nicotinamide glucosidiodide (I) and the corresponding orthodihydro compound (II) were obtained from Professor Paul Karrer (4). Nicotinamide nucleoside (III) was isolated from partially hydrolyzed coenzyme, and was placed at our disposal by Dr F Schlenk. Arecoline hydrobromide (IV) and piperine (V) were commercial samples. Compounds I to V were all used as received, although it is probable that Compound I was not entirely pure. Nicotinamide methochloride (VI) was prepared by direct methylation of nicotinamide.

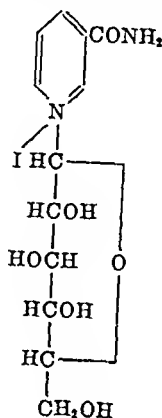
The preparation of Charcoal Adsorbate A has been previously described (2).

*Biological Assays*—Microbiological determinations of nicotinic acid and the precursor were carried out as before (2).

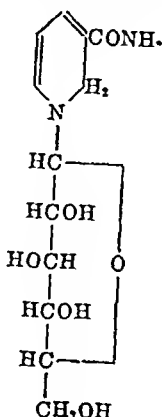
The basal ration used for the estimation of anti-blacktongue activity with dogs was that of Schaefer *et al* (5), except that 3 per cent of cod liver oil was replaced with corn oil and vitamins A and D were supplied through halibut liver oil. A norit eluate from solubilized liver was also

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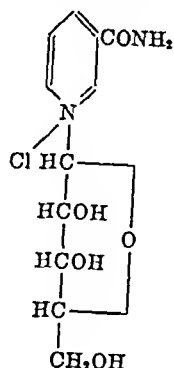
fed at a level of 1.25 cc per dog per day. This eluate was prepared essentially as described by Hutchings *et al* (6), and contained folic acid activity equivalent to 650 mg of solubilized liver per cc. The nicotinic acid contributed by this amount of norit eluate was negligible.



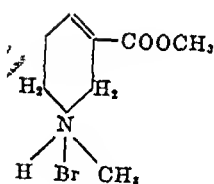
(I)



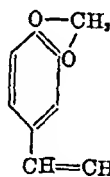
(II)



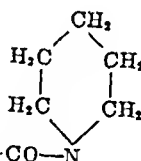
(III)



(IV)



(V)



(VI)

The ration was fed *ad libitum*, and each dog received in addition 100  $\gamma$  of thiamine chloride, 100  $\gamma$  of riboflavin, 60  $\gamma$  of pyridoxine hydrochloride, 500  $\gamma$  of calcium pantothenate, and 50 mg of choline chloride per kilo of body weight per day. The required amount of these vitamins was given by pipette twice weekly. Halibut liver oil was administered at the same times on the basis of 3 drops per kilo of body weight per week.

The end-point used to denote marked nicotinic acid deficiency was the appearance of typical blacktongue symptoms, such as inflammation of the gums, palatine redness, anorexia, and drastic loss of weight. The criteria of effectiveness for materials fed were blacktongue curative action and growth response. The growth response of all dogs used was standardized with 25 mg doses of nicotinic acid, and each dog served as its own control.

The chick assays for nicotinic acid activity were carried out according to the method of Briggs *et al* (7).

*Microbiological Assay of Compounds Related to Nicotinic Acid*—The results obtained when several compounds were assayed under the conditions used in the microbiological determination of free nicotinic acid and the precursor are collected in Table I. It is evident that the simple esters behave in much the same way toward alkali as does the precursor. The quaternary salts, Compounds I, II, and III, on the other hand, are somewhat less active after alkali treatment than before. The reason for this behavior is not apparent, since Compounds I and III should be hydrolyzed to free nicotinic acid and Compound II not affected at all by dilute alkali at room temperature (8).

*Dog Assays*—30 gm of Charcoal Adsorbate A, which according to microbiological assay carried 0.12 and 1.65 mg per gm of nicotinic acid and precursor respectively (2), were stirred for 1 hour at room temperature with 750 cc of 5 per cent ethyl alcoholic potassium hydroxide. The charcoal was filtered off, the eluate concentrated to a thick paste *in vacuo*, and neutralized with hydrochloric acid. The mixture was concentrated to dryness *in vacuo*, the residue extracted three times with 100 cc portions of 95 per cent ethanol, and the combined extracts again taken to dryness. The residue was taken up in 40 cc of 25 per cent ethanol. This solution gave a pronounced yellow color with cyanogen bromide and aniline, and by microbiological assay contained a total of 49.6 mg of nicotinic acid. Not more than 3.6 mg of this could have come from the free nicotinic acid of the original adsorbate.

A dog with blacktongue was given one-half of this preparation by pipette. Within 24 hours the animal gained 1050 gm and all symptoms of blacktongue were alleviated. The total weight increase resulting from the dose was about 98 per cent of the standard response to 25 mg of nicotinic acid (Dog 9, Table II).

In a subsequent experiment a similar eluate from Charcoal Adsorbate A, which by bacterial assay contained 27.5 mg of nicotinic acid, was mixed into the ration of Dog 9. The dog had been given 10 mg of pure nicotinic acid the previous day in order to overcome the anorexia of blacktongue. The response (Table II) again demonstrates that the alcoholic potassium hydroxide eluate possesses as much nicotinic acid activity toward the dog as toward *Lactobacillus arabinosus*.

In an effort to determine the nicotinic acid activity of the precursor itself, 15 gm of the same adsorbate were suspended in 100 cc of water and fed by stomach tube to Dog 8. The response obtained (Table II) indicated 39 per cent as much activity in the precursor as in a bacteriologically equivalent amount of nicotinic acid, and was much greater than could be accounted for by the small amount of free nicotinic acid in the preparation. Furthermore, control experiments demonstrated that the dog is unable to utilize free nicotinic acid adsorbed on charcoal (Table II).

TABLE I  
*Microbiological Assay of Compounds Related to Nicotinic Acid*

Compound assayed	Nicotinic acid activity	Activity after alkali treatment
	<i>per cent</i> †	<i>per cent</i> †
Ethyl nicotinate	0	94
Propyl     "	0	103
Butyl       "	23	93
I	13	6 7
II	22	11
III	104	91
IV	0	0
V	0	0
VI	0	0

\* Exposure to 0.25 per cent sodium hydroxide for 5 minutes at room temperature

† Per cent activity compared to pure nicotinic acid on an equimolar basis

TABLE II  
*Nicotinic Acid Activity of Various Materials for the Dog*

Dog No	Material fed	Nicotinic acid content*	Growth response	Nicotinic acid activity†
		<i>mg</i>	<i>gm</i>	<i>per cent</i>
8	Nicotinic acid	25	1450	100
	Charcoal Adsorbato A	26.6	600	39
	Nicotinic acid adsorbed on charcoal	23.4	Death	
9	Nicotinic acid	25	1850	100
	Vitab	26	2700	101
	+ nicotinic acid	10		
	Eluate from Charcoal Adsorbato A	27.5	3100	111
	+ nicotinic acid	10		
12	Eluate from Charcoal Adsorbato A	24.8	1800	98
	Nicotinic acid	25	1000	100
	Vitab	26.6	950	90
13	Nicotinic acid	25	900	100
	Vitab	26.6	950	99
	Nicotinic acid adsorbed on charcoal	23.4	(-500)	
16	Nicotinic acid	25	1100	100
	Alkali-treated vitab	26.6	1050	90

\* Total nicotinic acid content as determined microbiologically after alkali treatment

† Calculated by adjusting the observed growth response to the basis of 25 mg of total nicotinic acid activity in the preparation fed, and comparing with the response of the same animal to 25 mg of pure nicotinic acid

Several dog assays were also carried out on vitab. The results in Table II show that the nicotinic acid values found are close to those determined microbiologically after alkali treatment, and thus support the idea that the precursor is biologically active.

Assays of ethyl nicotinate were carried out with three different dogs, and in each case the ester proved to be fully as effective as an equimolecular amount of the free acid. This confirms the work of Woolley *et al.* (9).

*Chick Assays*—Ethyl, propyl, and *n*-butyl nicotines were also tested on chickens. The three esters in the order named showed 48, 55, and 80 per cent as much activity as equimolar amounts of nicotinic acid. Charcoal Adsorbate A and pure nicotinic acid adsorbed on charcoal were both

TABLE III  
*Nicotinic Acid Activity of Various Materials for the Chick*

Experiment No	Group No	Supplement to basal ration	Nicotinic acid content†	Growth at 4 wks
			mg	gm
1	1	None		180
	2	Nicotinic acid	0.5	184
	3	" "	1.0	210
	4	Charcoal Adsorbate A	1.0	176
	5	Nicotinic acid adsorbed on charcoal	0.93	155
2	6	None		201
	7	Nicotinic acid	0.5	238
	8	" "	1.0	244
	9	Charcoal Adsorbate A	1.0	162
	10	Nicotinic acid adsorbed on charcoal	0.93	208
	11	Alcoholic KOH eluate from Charcoal Adsorbate A	0.66	226

\* Five chicks were used in each group.

† Amount per 100 gm. of ration, as determined microbiologically.

inactive for the chick (Table III), and in fact tended to cause a slower growth than that of the control group. One chick assay was also carried out on an alcoholic potassium hydroxide eluate of Charcoal Adsorbate A. The growth response given in Table III indicates definite activity, but only about half as much as would be expected from the quantity of nicotinic acid found in this particular eluate by the bacterial method.

Chick assay of vitab, on the other hand, showed only 855  $\gamma$  of nicotinic acid per gm. before alkali treatment, and 2014  $\gamma$  per gm. afterwards. The corresponding values obtained microbiologically on this sample of vitab were 1530 and 1950  $\gamma$  per gm. respectively. Thus the total activities found agree very well, but the precursor content calculated from the chick results is 1159  $\gamma$  per gm. *versus* 420  $\gamma$  per gm. found with the bacteria.

## DISCUSSION

All of the evidence collected to date regarding the chemical behavior of the substance which is produced by mild alkali treatment of wheat extracts and which is measured by *Lactobacillus arabinosus* as nicotinic acid has been consistent with the hypothesis that the active factor so produced actually is this vitamin. To this evidence has now been added the observation that the hydrolytic product is fully effective as a source of nicotinic acid for dogs. Furthermore, it has now been demonstrated that certain compounds do exist, *eg* butyl nicotinate, which are only partially active on the bacteria and form nicotinic acid under the same conditions as those used in hydrolyzing the precursor.

The existence of the precursor as a derivative of nicotinic acid, therefore, seems to be definitely established. From the data now at hand, it also is possible to state with considerable assurance that the substance is able to serve as a source of nicotinic acid for the dog. The results of the dog assay both on vitab and on Charcoal Adsorbate A support this statement. The fact that the response in the latter case was only 39 per cent of that which might have been obtained is not surprising in view of the tenacity with which the precursor is held on active carbon (2).

The chick assay on vitab is interesting because it indicates a much higher precursor content than had previously been found by the bacterial method. This may be interpreted to mean that the chick is entirely unable to utilize the precursor in aqueous solution. If this is actually the case, it is merely another example of the greater ability of the dog to utilize derivatives of nicotinic acid. Ethyl nicotinate furnishes another example of the same sort. If the precursor content of vitab as determined by the chick method is accepted as the true value, the results by dog assay on this material must be regarded as a very strong indication that the precursor is completely utilized by the dog.

The above result by chick assay on vitab also provides further support for the hypothesis advanced previously (2) that *Lactobacillus arabinosus* utilizes the precursor to a certain extent when it is in solution. In this connection it should be emphasized again that the existing figures on the amounts of the precursor present in biological materials are probably much too low because, first, there is no assurance that the extraction methods used remove all the precursor from the sample, second, more or less hydrolysis no doubt occurs during the extraction, and third, the differential microbiological assay may not indicate the total amount of precursor in the extract. It is thus quite possible that, in certain tissues at least, the bulk of the nicotinic acid exists in the form of the precursor.

The microbiological assay on the quaternary salts, Compounds I, II, and III, before and after alkali treatment indicate that the precursor is

not a compound of this general type. This conclusion is in agreement with that reached from a study of the cyanogen bromide reaction (2).

The results of the present study have certain obvious implications relative to methods used for the determination of nicotinic acid. The microbiological assay should not be applied to extracts prepared with cold or hot water or under conditions of acid treatment as mild as or milder than 0.1 N hydrochloric acid at 100°, if the total nicotinic acid value is desired. Autoclaving the sample with 1 N sodium hydroxide, 1 N hydrochloric acid, or 1 N sulfuric acid, on the other hand, should be entirely satisfactory. It would probably be safest to use similarly drastic extraction procedures when the chemical method of estimation is to be employed. Although the cyanogen bromide reaction probably measures the precursor, there is no assurance at the present time that the result necessarily is an accurate indication of its nicotinic acid content.

#### SUMMARY

The existence of an alkali-labile precursor of nicotinic acid in certain natural products has been confirmed. It is very probable that the dog can utilize the precursor as a source of nicotinic acid.

Several sugar derivatives of nicotinamide do not resemble the precursor in their action on *Lactobacillus arabinosus*. On the other hand, several simple esters of nicotinic acid exhibit a type of biological activity closely similar to that of the precursor.

The authors wish to express their deep appreciation to Professor Paul Karrer and Dr. F. Schlenk for the gift of several compounds, and to T. D. Luckey and G. Briggs for assistance in carrying out the chick assays.

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# THE USE OF LACTOBACILLUS ARABINOSUS IN THE MICROBIOLOGICAL DETERMINATION OF PANTOTHENIC ACID

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Various microbiological procedures, utilizing *Lactobacillus casei* (1-3), *Saccharomyces carlsbergensis* (4), or *Protocus morgani* (5) as the test organism, have been described for the determination of pantothenic acid. Snell and Wright (6) reported that pantothenic acid was required as a growth factor for *Lactobacillus arabinosus* and that the determination of pantothenic acid with this organism was possible. Recently Hoag *et al.* (7) have proposed the use of *L. arabinosus* in an assay for pantothenic acid. They employed a basal medium in which the pantothenic acid present in several naturally occurring materials had been inactivated by alkali. This communication presents our observations on the use of *L. arabinosus* for the microbiological determination of pantothenic acid by a modification of the Snell and Wright medium in which pantothenic acid, rather than nicotinic acid, is the limiting factor.

## Procedure

The organism used is *Lactobacillus arabinosus* 17-5. It is carried by monthly transfer in 1 per cent yeast extract, 1 per cent glucose, 1.5 per cent agar. Inoculum tubes are prepared either by direct transfer from stock culture or by daily transfer in 1 per cent yeast extract, 1 per cent glucose broth. After incubation for 24 hours at 33°, the culture is centrifuged, and the cells are resuspended in physiological saline. From this dense suspension a second, very light, suspension is prepared with which the test is inoculated.

The basal medium is shown in Table I. We have found it convenient to prepare at one time 5 liters of a stock solution of double strength medium containing all the ingredients listed with the exception of glucose and the synthetic vitamins. The solution may be preserved indefinitely under benzene at room temperature without previous heat sterilization. A stock vitamin solution is also prepared, containing in each 100 ml., thiamine chloride, riboflavin, nicotinic acid, 4 mg. of each, pyridoxine hydrochloride, 8 mg., *p*-aminobenzoic acid, 0.4 mg., and biotin, 10  $\gamma$ . The vitamin supplement, in a dark bottle, is stored in the refrigerator without added preservatives. It is renewed at frequent intervals.

A solution containing 100  $\gamma$  per ml of calcium pantothenate also is prepared. To determine the response of the organism to known amounts of pantothenic acid (Fig 1) this solution is diluted and distributed in amounts equivalent to 0.02 through 0.20  $\gamma$  per tube. Various amounts of the materials to be assayed, estimated to contain between 0.01 and 0.20  $\gamma$

TABLE I  
Basal Medium

	Per 100 ml medium (double strength)		Per 100 ml medium (double strength)
Cascin,* gm	1.0	Inorganic Salts B (6), ml	1.0
Cystine, mg	20.0	Glucose, gm	4.0
Tryptophane, mg	20.0	Thiamine chloride, $\gamma$	200.0
Sodium acetate (anhydrous), gm	1.2	Riboflavin, $\gamma$	200.0
Adenine, mg	1.0	Nicotinic acid, $\gamma$	200.0
Guanine, "	1.0	Pyridoxine hydrochloride, $\gamma$	400.0
Uracil, "	1.0	p-Aminobenzoic acid, $\gamma$	20.0
Xanthine, mg	1.0	Biotin, $\gamma$	0.5
Inorganic Salts A (6), ml	1.0	pH adjusted to 6.6-6.8	

\* Hydrochloric acid hydrolyzed, norit treated, vitamin free cascain is employed (8)

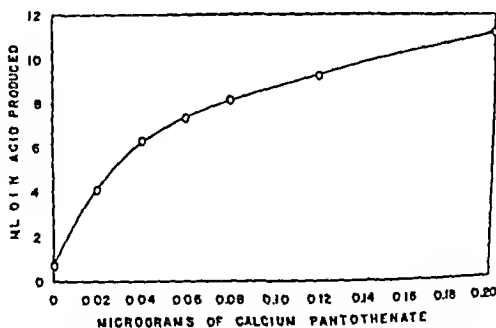


FIG 1 Response of *Lactobacillus arabinosus* to added pantothenic acid

of pantothenic acid, and to cover a 5-fold range, are pipetted into additional tubes. All tubes are then diluted to 5 ml with distilled water.

The standard tubes and those containing the test materials receive 5 ml each of double strength medium. The tubes are then plugged with cotton and autoclaved at 15 pounds for 15 minutes. The test is seeded with 1 drop per tube of the inoculum previously described. After 72 hours

incubation at 33° the amount of acid produced is determined by titration with 0.1 N NaOH. Brom thymol blue is used as the indicator.

### Results

In order to have a basis for evaluating the procedure described, a number of materials have been assayed in triplicate by this new technique, that of Landy and Dicken (2), and also that of Pennington *et al* (1). The proposed procedure gave values for the pantothenic acid content of the various materials tested which were in essential agreement with the results obtained by the other methods employed (Table II). Generally, the results obtained at varying assay levels were in better agreement with each

TABLE II  
Comparative Pantothenic Acid Assays on Various Materials

Material	Landy and Dicken <i>Lactobacillus</i> cases	Pennington Snell and Williams <i>Lactobacillus</i> cases	<i>Lactobacillus</i> <i>arabinosus</i>
	$\gamma$ per gm or ml	$\gamma$ per gm or ml	$\gamma$ per gm or ml
Yeast extract	321	326	306
Proteose peptone	26	26	23
Bacto peptone	2.7	2.0	3.4
Liver powder 1:20	329	314	324
Grass juice powder	42	47	47
Tryptone	6.2	7.5	6.6
Valentine's meat extract	12.0	15.0	13.4
Soy bean peptone	13.0	13.5	13.2
Cottonseed peptone	11.6	11.3	11.5
Malt extract	3.5	3.9	3.8
Urine	6.5	6.1	8.7

other when the materials were assayed by the *Lactobacillus arabinosus* method than when assayed by either of the other two procedures studied.

Quantitative recovery of pantothenic acid ranging from 92 to 106 per cent was obtained when known amounts of pantothenic acid were added directly to the test materials being assayed by the procedure described (Table III).

The practicability of the procedure presented here as a rapid turbidimetric method for pantothenic acid determination was investigated. A group of materials was tested in duplicate. In one test the comparative densities after 18 hours incubation were determined in the Klett-Summerson photoelectric colorimeter. The other group was titrated with 0.1 N NaOH in the usual manner after 72 hours. Values obtained by turbidimetric comparison agreed within a narrow range with those obtained by titration (Table IV).

TABLE III  
*Recovery of Pantothenic Acid*

Material	Pantothenic acid in material	Pantothenic acid added	Pantothenic acid found	Pantothenic acid calculated	Per cent recovery
	$\gamma$ per tube	$\gamma$ per tube	$\gamma$ per tube	$\gamma$ per tube	
Yeast extract	0 030	0 040	0 067	0 070	96
	0 045	0 060	0 107	0 105	102
	0 075	0 100	0 186	0 175	106
Proteose peptone	0 014	0 040	0 050	0 054	93
	0 021	0 060	0 078	0 081	96
	0 035	0 100	0 128	0 135	95
Rat liver	0 010	0 040	0 046	0 050	92
	0 015	0 060	0 070	0 075	93
	0 025	0 100	0 125	0 125	100
Urine	0 004	0 040	0 042	0 044	96
	0 006	0 060	0 063	0 066	95
	0 010	0 100	0 106	0 110	96

TABLE IV

*Comparison of Pantothenic Acid Values by Turbidimetric and Titration Methods*

Material	Turbidimetric	Titration
	$\gamma$ per gm or ml	$\gamma$ per gm or ml
Liver powder 1 20	341	325
Proteose peptone	27	25
Rat liver (pantothenic acid deficient rat)	35	36
Urine	8 4	8 7
Grass juice powder	51	45
Yeast extract	270	301

TABLE V

*Effect of Oleic Acid on Response of Lactobacillus arabinosus to Pantothenic Acid*

Material	Pantothenic acid content with out oleic acid added to sample	Pantothenic acid content with oleic acid added to sample	Material	Pantothenic acid content with out oleic acid in medium	Pantothenic acid content with oleic acid in medium
	$\gamma$ per gm or ml	$\gamma$ per gm or ml		$\gamma$ per gm or ml	$\gamma$ per gm or ml
Proteose peptone	25	69	Proteose peptone	27	27
Yeast extract	285	561	Yeast extract	304	309
Urine	8 5	22	Urine	11	7 7
Malt extract	7 0	13	Rat liver (pantothenic acid deficient rat)	40	33

Bauernfeind *et al* (9) and Neil and Strong (10) have reported the stimulatory effect of various fatty acids on the growth of *Lactobacillus casei* in the presence of suboptimal amounts of pantothenic acid. We found *Lactobacillus arabinosus* usually responded in a similar manner to the addition of oleic acid. Increasing amounts of oleic acid when added to a constant suboptimal amount of pantothenic acid gave increased acid production with an apparent maximum stimulation after 400  $\gamma$  per 10 ml culture tube of oleic acid had been added.

Several natural materials containing 25  $\gamma$  of added oleic acid per ml of final dilution gave an apparent pantothenic acid content of more than twice the amount present when the same materials were assayed in the absence of added oleic acid (Table V). The addition of oleic acid to the culture medium in sufficient amounts so that each tube, standard and test material alike, received approximately 500  $\gamma$  effectively equalized the stimulatory effect of the fatty acid in such a way that pantothenic acid values thus obtained were consistent with those obtained in the absence of oleic acid (Table V).

#### DISCUSSION

From the results presented it is evident that *Lactobacillus arabinosus* may be used as the test organism in the determination of pantothenic acid with a modification of the Snell and Wright medium. It should be noted that certain slight changes have been made in the basal medium. The glucose has been increased to 2 per cent. The B vitamins have been substantially increased and *p*-aminobenzoic acid has been included in the vitamin supplement. If it is desired to increase the buffer capacity of the medium, the amount of sodium acetate may be increased.

By use of the technique described we obtained pantothenic acid values which agreed well with those found by assay with two of the more commonly used microbiological procedures.

Among the advantages of the proposed procedure is the fact that it is accurate as an 18 hour turbidimetric method. The Landy and Dicken method with *Lactobacillus casei* cannot be so modified, since the medium lacks the *Lactobacillus casei*-stimulating factors (11) and gives non-specific results at 18 to 24 hours. With the Pennington, Snell, and Williams procedure, it frequently is difficult to obtain low blanks. This probably is due to the difficulty of destroying the pantothenic acid in the natural materials employed without simultaneous destruction of the other stimulatory and essential *L. casei* factors. Recent studies on the growth factor requirements of *L. arabinosus* have shown them to be much simpler than those of *L. casei* (12, 13). The use of *L. arabinosus*, therefore, has the distinct advantage of giving a more specific response to pantothenic acid,

since there are fewer factors which stimulate growth, and since the organism requires a less complex medium

The release of pantothenic acid from the combined forms to make it available for *Lactobacillus casei* has been investigated thoroughly by others (10, 14). Therefore, no effort has been made to study the availability of combined pantothenic acid by this method, since the results indicated that, in the materials studied, pantothenic acid was equally available to both *L. casei* and *L. arabinosus*.

Like *Lactobacillus casei*, we found *Lactobacillus arabinosus* to be stimulated by the addition of fatty acids (using oleic acid as an example) in the presence of suboptimal amounts of pantothenic acid. The effect of this stimulation may be overcome by the inclusion of oleic acid in the basal medium (see Table V), or by ether extraction of the sample prior to assay for pantothenic acid (10).

#### SUMMARY

A method has been presented for the microbiological determination of pantothenic acid by use of *Lactobacillus arabinosus* 17-5. Various materials, assayed by this method, gave pantothenic acid values which compared favorably with those obtained by assay methods previously described. The advantages of this method over existing pantothenic acid methods have been discussed.

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# THE SOURCE OF THE EXTRA LIVER FAT IN VARIOUS TYPES OF FATTY LIVER\*

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The quantity of fatty acids in the liver may be regarded as the resultant of several processes all of which are presumed to be proceeding in the normal animal (Fig. 1). Any variation in the quantity of liver fatty acids, in response to variation of experimental conditions, may be assigned to a change in rate of one or another of these processes. There is ample evidence, for instance, that the fatty liver encountered in routine human autopsy material results preponderantly from excessive migration of fatty acids from the depots to the liver, Process *c* (1). The fatty liver that is seen when thiamine is fed to rats on a high carbohydrate diet (2) may be assigned to an acceleration of fatty acid synthesis from carbohydrate precursors, Process *a*. That thiamine does indeed stimulate this process has recently been proved with the aid of isotopes (3).

Barrett, Best, and Ridout (4) have investigated the source of extra liver fat in various types of fatty liver by the expedient of labeling the depot fatty acids with deuterium. When fatty liver is provoked in an animal after such preliminary treatment, if the process is exclusively one of migration of depot fatty acids to the liver (Process *c*), the deuterium concentration in the liver fatty acids will drop to, but not below, the corresponding value in the depot fatty acids. If, on the other hand, a portion of the new liver fat is derived from the diet (Process *a* or *b*), the deuterium concentration in the liver fatty acids would be expected to fall below that in the depot fatty acids. On the basis of such experiments the fatty liver resulting from an alipotropic high carbohydrate diet was ascribed to accumulation of non-isotopic fatty acids, presumably synthesized from dietary carbohydrate. The fatty liver incident to the injection of anterior pituitary extract into fasting mice, however, proved to be the result of migration of labeled depot fatty acids to the liver.

The experimental procedure followed in the present paper is a counterpart of that employed by Barrett *et al.* (4). Instead of labeling the preformed

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depot fatty acids, we have labeled the newly synthesized fatty acids with deuterium. This has been accomplished by raising the level of the body fluids of animals on fat-free diets with respect to  $D_2O$ . Under these conditions, each newly synthesized molecule of fatty acid will acquire deuterium from the body fluids (5). Whereas the exact concentration of deuterium in the newly synthesized fatty acids is a matter of some disagreement (6-8), it appears to be nearly equal to one-half the concentration in the body fluids, and this figure will be assumed in subsequent calculations. Thus from the quantity of fatty acid, its deuterium concentration, and the deuterium concentration of the body water, it is possible to estimate the quantity of newly synthesized fatty acid, that is, the fatty acid synthesized during the period of deuterium oxide administration.

Two types of fatty livers in rats have been studied. In one series of experiments fatty liver was induced by withholding choline from the diet.

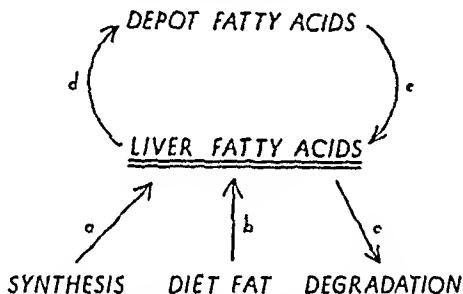


FIG. 1 The sources and fates of liver fatty acids

for varying periods of time. In another series, advantage was taken of the well known antilipotropic action of cystine. In these experiments, the period of  $D_2O$  administration was 4 days. In an additional experiment, the fatty liver resulting from the injection of anterior pituitary extract into fasting mice was studied.

#### EXPERIMENTAL

The basal diet in the experiments with rats contained 10 per cent of casein (Labco, vitamin-free), 84 per cent of glucose monohydrate, 4 per cent of salt mixture (No. 2, U S P XII), and 2 per cent of roughage (Celluration). To each kilo of this diet were added 6.25 mg of thiamine hydrochloride, 5 mg each of riboflavin and pyridoxine, and 25 mg each of niacin and calcium pantothenate. Each animal received in addition 1700 units of vitamin A and 200 units of vitamin D weekly, and 0.2 cc of linseed oil every 10 days.

In the studies of the effect of choline, 28 to 30 day-old male rats of the

Sherman strain, weighing 45 to 55 mg, were used. Choline chloride, 0.5 per cent, was added to the diet of the control groups. Diet and drinking water were offered *ad libitum* throughout the experiment. After a preliminary period of 5 to 15 days, 1 cc of 99.5 per cent  $D_2O$  was injected subcutaneously per 100 gm of body weight, and the drinking water replaced by 2.5 per cent  $D_2O$ . 4 days later the animals were sacrificed, livers and eviscerated carcasses pooled, fatty acids isolated, and these, together with samples of body water, analyzed for D (9).

In the studies of the effect of cystine, the same basal diet was employed. To the diets of both groups, 0.0625 per cent of choline chloride was added, and, in addition, 0.5 per cent of *L*-cystine was added to the diet of the experimental group. After a 10 day preliminary period,  $D_2O$  was administered subcutaneously and by mouth for 4 days, and the animals worked up as described above.

The effect of anterior pituitary substance was studied in female adult mice weighing 21 to 22 gm each.  $D_2O$  was administered in the same fashion as in the experiments with rats. From the moment of injection of heavy water, all food was withheld. After 16, and again after 22 hours, 1 cc of anterior pituitary extract (10),<sup>1</sup> in 1.5 per cent  $D_2O$ , was injected subcutaneously into each of the five experimental animals. 29 hours after the initiation of the fast, the mice were killed. The six control mice were simply given  $D_2O$  and fasted for 29 hours.

#### DISCUSSION

In each pair of experiments in which the action of choline was studied (Table I), the choline-deficient rats gained less in weight, and had less fatty acid in their depots and more in their livers than did the control rats. Of the "extra" fatty acid that was found in the livers of the choline-deficient animals, about one-half could be accounted for as "new" fatty acid, synthesized in the last 4 days of life. It will further be noted that, whereas the quantity of the new fatty acid in the livers of these animals was appreciably greater than in the control series, the quantity of new fatty acid in the depots of the deficient rats was equal to or less than that recovered from the control animals. In the control groups, the depots contained from 12 to 18 times as much new fatty acid as the livers, while in the choline-deficient groups, only 3 to 7 times as much new fatty acid was found in the depots as in the livers.

These findings are in agreement with the picture that the major site of fatty acid synthesis is the liver, and that in choline deficiency the products of this synthesis do not escape to the depots as rapidly as in normal animals.

<sup>1</sup> The authors are indebted to Dr. R. Weil for assistance in the preparation and standardization of this extract.

Considered together with the earlier finding that the rate of catabolism of fatty acids is normal in the choline-deficient animal (11), it is concluded that choline deficiency interferes with Process *d*, Fig 1 Since the adminis-

TABLE I

*Effect of Choline Deficiency upon Distribution of Newly Synthesized Fatty Acids*

Six groups of three young male rats each were kept for preliminary periods of 5, 10, and 15 days on the diet described in the text To the diet of the three control groups was added 0.5 per cent of choline chloride After the preliminary period the concentration of  $D_2O$  in the body fluids was abruptly raised and maintained at an elevated level for 4 days Fatty acids were then isolated from liver and depot fat and analyzed for deuterium

Duration	Diet	Weight gain per rat per day	Fatty acids per rat		Liver fatty acids	Depot fatty acids	Body water	New fatty acids per rat	
			Liver (a)	Depot (b)				Liver (f)	Depot (g)
days		gm	gm	gm	atom per cent D	atom per cent D	atom per cent D	gm	gm
9	Choline free	0.9	0.353	2.775	0.313	0.195	1.08	0.20	1.00
	Control	1.6	0.126	3.098	0.338	0.169	1.06	0.08	0.99
14	Choline free	1.2	0.456	2.925	0.202	0.076	1.05	0.18	0.42
	Control	1.9	0.092	4.931	0.274	0.102	1.11	0.05	0.91
19	Choline free	1.6	0.455	3.653	0.285	0.237	1.29	0.20	1.35
	Control	2.2	0.150	5.357	0.418	0.176	1.22	0.10	1.55

\* The quantities of newly synthesized fatty acids in liver and depot fat were calculated from the analytical values  $f = 2ae/c$ ,  $g = 2bd'e$

TABLE II

*Effect of Dietary Cystine upon Distribution of Newly Synthesized fatty Acids*

Two groups of three young male rats each were kept for 10 days on the diets described in the text The one diet contained 0.5 per cent of added L-cystine The body fluids of the rats were then enriched with respect to  $D_2O$  for 4 days Fatty acids were isolated from liver and depot fat and analyzed for deuterium

Diet	Food consumed per rat per day	Weight gain per rat per day	Fatty acids per rat		Liver fatty acids	Depot fatty acids	Body water	New fatty acids per rat	
			Liver	Depot				Liver	Depot
	gm	gm	gm	gm	atom per cent D	atom per cent D	atom per cent D	gm	gm
Cystine	8.1	2.2	0.324	4.553	0.439	0.308	1.25	0.23	2.24
Control	6.0	1.0	0.123	3.665	0.388	0.281	1.29	0.07	1.60

tration of thiamine, in producing fatty livers (2, 3), does so by stimulus of Process *a*, it is only in a very superficial sense that choline and thiamine may be considered as antagonists

In the study of the antilipotropic action of cystine (Table II) the rats

receiving supplementary cystine had more fatty acid not only in their livers but also in their depots. They consumed more food and gained more in weight. As was the case with the choline-deficient animals, the rats receiving cystine had more new fatty acid in their livers than did the control rats. In contrast to choline deficiency, however, there was an appreciably greater quantity of new fatty acid in the depots of the cystine-fed rats than in the control series.

The rats receiving cystine developed fatty livers, as did the choline-deficient rats, because of an excessive accumulation of newly synthesized fatty acids. In this case, however, the accumulation cannot be ascribed to an interference with transport of liver fatty acids to the depot, as more of the labeled fatty acid was thus transported than in the control group. The fatty liver must therefore be assigned to excessive fatty acid synthesis, Process *a*, Fig. 1, such that the transportation mechanism, even operating

TABLE III

*Effect of Anterior Pituitary Extract upon Distribution of Newly Synthesized Fatty Acids*

Eleven fasting adult, female mice were given  $D_2O$  over a period of 29 hours. Anterior pituitary extract was injected into five of these animals at the 16th and again at the 22nd hour. Fatty acids were isolated from liver and depot fat and analyzed for deuterium.

	Fatty acids per mouse		Liver fatty acids	Depot fatty acids	Body water	New fatty acids per mouse	
	Liver	Depot				Liver	Depot
	gm	gm	atom per cent $D$	atom per cent $D$	atom per cent $D$	gm	gm
Anterior pituitary	0.227	0.808	0.032	0.024	1.89	0.008	0.02
Control	0.081	0.805	0.025	0.019	1.70	0.002	0.02

at capacity, is overloaded. This conclusion is in accord with the views of Mulford and Griffith (12) that the addition of cystine to a diet relatively poor in the sulfur amino acids improves its nutritive value.

Cystine and choline have appeared to be antagonists in that they affect the fat content of the liver in opposite directions. That this was not a simple antagonism was suspected from the fact that the composition of the liver lipids in choline deficiency was not the same as that observed when cystine was fed (13). With reference to Fig. 1, this difficulty is now resolved by assigning the function of choline to Process *d*, and that of cystine to Process *a*.

When fatty liver was provoked in mice by the injection of anterior pituitary substance (Table III), the analytical results obtained were entirely different from those obtained in the foregoing experiments. As the procedure was of very short duration and as no food was allowed, very

little new fatty acid was synthesized. About 150 mg more fatty acid was found in the livers of the injected animals than in those of the controls. Only 8 mg of this could be accounted for as new fatty acid. The rest must have been preexisting depot fatty acid which had migrated to the liver as a result of the anterior pituitary extract administered. The effect of the injection in promoting fatty liver must therefore have been a stimulus to Process *c*, Fig 1. This finding is in complete accord with the findings of Barrett, Best, and Ridout (4).

It should be pointed out that the bulk of the evidence dealing with human fatty liver indicates that this disease arises from a process fundamentally similar to that observed in the mice receiving anterior pituitary extract, that is, excessive mobilization of depot fat and migration to the liver.

It is suggested that the various types of fatty liver may with advantage be classified with respect to the source of the extra fat found in the liver.

#### SUMMARY

The source of the extra liver fatty acids in rats on an aliprotic diet, rats fed cystine, and mice injected with anterior pituitary substance has been studied with the aid of deuterium.

The fatty liver of choline deficiency has been shown to result from increased transportation of fatty acids from liver to depots. The fatty liver following the feeding of cystine, like that incident to thiamine administration, is the result of increased rate of fatty acid synthesis. The fatty liver that follows the injection of anterior pituitary substance into fasting mice is the result of excessive mobilization of depot fat and migration to the liver. This latter process is most nearly like that which appears to cause the human fatty livers seen in routine autopsy material.

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## CITRIC ACID DETERMINATION

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Three types of methods have been proposed for the determination of citric acid in urine. Salant and Wise (1) used the unspecific mercuric sulfate reagent of Denigès (2) in a study of the toxicity of citrates. Thunberg (3) developed an enzymatic method employing a dehydrogenase said to be specific for citrate. The remaining methods depend on the so called pentabromoacetone method of Stalhe (4), a qualitative test published in 1897 which Kunz (5), in 1914, used as the basis of the first quantitative pentabromoacetone method. It has been generally overlooked that in 1847 Cahours (6) prepared a bromination product from several citrates which he called "bromoxaforme" which according to his melting point and solubility data is without doubt identical with pentabromoacetone. Cahours was aware of the specificity and analytical applicability of the reaction. ("Le brome peut donc servir à reconnaître de petites quantités d'acide citrique mélangées à l'acide tartrique"). Kunz's technique was adapted for use with urine by Amberg and McClure (7). Fasold (8) with this method was the first to isolate citric acid from normal urine.

Kometiani's (9) modification of this method was applied to urine by Sullmann and Schaerer (10). Pucher, Sherman, and Vickery (11) developed a spectrophotometric method based on the color formed when pentabromoacetone is treated with sodium sulfide. Our procedure is another modification of this method. It is precise, accurate, and time-saving. The "pentabromoacetone reaction" has been clarified and new reagents with certain advantages have been introduced. While the procedure was designed for 15 cc samples of urine, it is also applicable to 2 cc samples, as well as to other fluids of comparable citrate content. The range is from 1 to 40 mg, although with care, useful results are obtainable down to 0.2 mg. The method has already been used in a study of the variation of urinary citrate with the menstrual cycle (12).

### Principle

The citric acid is oxidized by manganese dioxide, in the presence of bromine, to acetonedicarboxylic acid, which is then rapidly brominated with simultaneous decarboxylation, yielding pentabromoacetone. After reduction of the excess manganese dioxide and bromine with hydrazine,

the pentabromoacetone is isolated by extraction with petroleum ether. It is then treated with a sulfite solution, which we find destroys pentabromoacetone smoothly and rapidly, liberating all 5 bromine atoms as bromide ions. The bromide is determined by a direct argentometric titration.

*Reagents—*

27 N sulfuric acid 1500 cc of concentrated acid, diluted to 2 liters

2 N bromide-bromate 85.7 gm of NaBr and 28 gm of NaBrO<sub>3</sub> (11 per cent excess) are dissolved and diluted to 500 cc

2 N silver nitrate 85.0 gm dissolved and diluted to 250 cc

10 N hydrazine 32.5 gm of N<sub>2</sub>H<sub>4</sub>, H<sub>2</sub>SO<sub>4</sub> are dissolved with the aid of 49 cc of 20 per cent NaOH or the equivalent and diluted to 100 cc

2 N manganese sulfate 111.5 gm of MnSO<sub>4</sub> · 4H<sub>2</sub>O dissolved and diluted to 250 cc

0.2 N KMnO<sub>4</sub> 31.6 gm dissolved and diluted to 1 liter

Petroleum ether (b.p. 35–60°) Preferably purified by shaking several times with concentrated sulfuric acid and distilling. A small forerun and residue are discarded.

0.01 per cent aqueous methylene blue solution

Sulfite reagent 26.0 gm of NaHSO<sub>3</sub> (halogen-free, for microanalysis) are dissolved in water and somewhat less than 40 cc of clean 20 per cent NaOH or the equivalent, and 50 cc of alcohol added. Dilute to 500 cc after checking the pH, which should be 7.4 to 7.8. If the pH is lower, add alkali as necessary. Keep in the refrigerator in several small bottles filled to the brim.

18 N H<sub>2</sub>SO<sub>4</sub> 100 cc of concentrated sulfuric acid diluted to 200 cc

25 per cent ferric alum 50 gm of FeNH<sub>4</sub>(SO<sub>4</sub>)<sub>2</sub> · 12H<sub>2</sub>O are dissolved with the addition of 7 cc of 27 N H<sub>2</sub>SO<sub>4</sub> and diluted to 200 cc

0.002 N KCNS Diluted from a suitable stock solution

Exactly 0.04 N silver nitrate 13.591 gm of pure silver nitrate are dissolved, 130 cc of nitric acid added, and the solution diluted to 2 liters

*Procedure*

*Urine*—A 24 hour specimen is collected and 25 cc of 27 N sulfuric acid are added as a preservative.

*Preliminary Bromination and Clarification*—25 or 30 cc of urine are placed in a 50 cc volumetric flask, followed by 10 cc of 27 N sulfuric acid and 2 cc of bromide-bromate mixture. The flask is set aside in the dark for 10 minutes, at room temperature, then 2 cc of 2 N silver nitrate are added, and the flask well shaken. The residual bromine is discharged by the minimum of solid metabisulfite or other sulfite preparation, and the solution is made to volume. If necessary, a fraction of a drop of

octyl alcohol may be used. The flask is shaken vigorously and the contents filtered into a dry 125 cc separatory funnel. The filtrate is shaken for 3 minutes with 10 cc of petroleum ether and a 25 cc aliquot transferred to a 60 cc separatory funnel to which are also added 1 cc of the manganese sulfate reagent and 1 cc of bromide-bromate mixture.

*Oxidation to Pentabromoacetone*—Permanganate is now added dropwise from a pipette with a tip drawn out to deliver about 60 drops per cc, with constant swirling to avoid local excess, until the orange color of the bromine is replaced by a brown color (about 1 to 3 cc). If at this point, depending upon the other substances in the solution, the color deepens and a slight precipitate of manganese dioxide slowly appears, or if the color becomes lighter, more permanganate is added. The funnel is allowed to stand for 20 minutes, when hydrazine is added dropwise to reduce the manganese dioxide and bromine (about 6 drops). The colorless fluid is shaken for 3 minutes with 10 cc of petroleum ether and the aqueous phase is discarded. The stopper, mouth, and walls of the funnel are washed twice with a jet of water from a wash bottle and the washings discarded. 20 cc of water are added, the funnel is shaken for about 20 seconds, and the water discarded without first swirling the funnel. This allows a small amount of insoluble, slimy material (usual in urine) to be deposited in a thin film on the walls of the funnel instead of collecting at the apex where it is more troublesome to handle.<sup>1</sup> 1 or 2 cc of methylene blue solution are allowed to flow around the walls of the funnel to permit easy observation of the interface. After a minute or two for drainage, the stem of the funnel is washed with water and the methylene blue layer very carefully drawn off until the petroleum ether enters and just fills the bore of the stop-cock. The water in the stem is sucked out by means of a capillary pipette and the petroleum ether is run into a 4 × 1 inch Pyrex test-tube. The funnel is washed twice with 1 cc portions of petroleum ether, the washings being added to the tube. A tiny fragment of porcelain is placed in the tube and the solvent evaporated to about 0.5 cc. The remainder of the solvent is allowed to evaporate at room temperature by holding the tube almost horizontally and rotating it so that the petroleum ether is spread over the bottom third in a thin film. This procedure prevents the loss of the somewhat volatile pentabromoacetone.

Since evaporation of the petroleum ether must be conducted without

<sup>1</sup> An alternative procedure is available from this point if preferred. 4 cc of sulfite solution are placed in the funnel which is shaken for 3 to 5 minutes. The sulfite is then transferred to a 4 × 1 inch test-tube and the funnel washed three times with 1 cc portions of added water. The tube is then immersed in a boiling water bath for 3 minutes, the 18 N sulfuric acid added, and the rest of the procedure followed as described below.



overheating because of the volatility of pentabromoacetone, we have devised for this purpose a heating spiral made from about 6 cm of nichrome wire cut from a standard replacement unit, which with the rest of the unit has the appropriate resistance for use with a 110 volt source. The heater uses about 6 watts with an evaporation time of about 7 minutes.

*Decomposition of Pentabromoacetone*—The wall of the tube is carefully washed with 2 cc of alcohol, and 4 cc of the sulfite solution are added immediately with mixing. The tube is immersed in a boiling water bath and 3 minutes later 1 cc of 18 N sulfuric acid is added cautiously, the tube is removed, and its contents are boiled over a free flame for a few seconds to expel sulfur dioxide. 0.5 cc of ferric alum are added and the solution is boiled for a few more seconds and cooled, when 1 cc of 0.002 N potassium thiocyanate is added and the titration carried out with 0.4 N silver nitrate. The end-point is recognized by the abrupt disappearance of the pink color and the appearance of a greenish tinge. As the end-point is approached, the tube is swirled vigorously, when the pink color is seen to return, presumably because of the bromide ion which has been absorbed by the precipitate. The equivalence point is not reached until the greenish tinge is seen to persist after vigorous swirling.

*Abnormal Urines*—The procedure described above is designed for human urines. With other materials certain changes may be necessary or desirable. Pure citrate solutions, of course, need not be subjected to preliminary bromination or clarification. In other cases the preliminary bromination may be omitted only when experience with many samples of the material shows that this simplification is permissible. It is only necessary to treat the petroleum ether extract from the preliminary bromination exactly as the second extract is treated and determine whether any appreciable volume of silver nitrate is used up.

*Procedure for Pure Citrate Solutions*—A sample containing preferably 5 to 15 mg of citric acid is placed in a 60 cc separatory funnel and diluted to 20 cc. 5.4 cc of 27 N sulfuric acid are added if the sample is approximately neutral. If, however, the sample is strongly acid or alkaline, the sulfuric acid is accordingly decreased or increased. The analysis is then completed as usual after addition of 1 cc of bromide-bromate, 1 cc of manganese sulfate, etc.

*Protein*—Urines containing protein may be treated with solid trichloroacetic acid and the filtrate used as usual. In samples containing large amounts of protein, a suitable amount is taken in a 50 cc volumetric flask, diluted to the mark with trichloroacetic acid solution, and an aliquot of the filtrate taken for analysis.

*Iodine*—In one case in which a patient had been receiving Lugol's solution the petroleum ether extract containing the pentabromoacetone

showed a violet tinge. This was removed by adding a few mg of sodium arsenite to the wash water.

**Glucose**—As noted by Reichard (13), low results are ordinarily obtained in the presence of glucose. If, before analysis, the specimen is not known to contain sugar, its presence will be indicated by the unusually high permanganate consumption. The following procedure yields quantitative results even in the presence of 100 times as much glucose as citric acid. A small sample is taken, 1 to 2 mg instead of 5 to 15. Bromination is carried out at  $0^{\circ}$  instead of at room temperature, with a corresponding increase in reaction time. At  $24^{\circ}$  the reaction time is about 20 minutes, whereas at  $0^{\circ}$  it is 4 to 5 hours. Manganese dioxide suspension is substituted for permanganate and the bromide-bromate is increased to 2 cc.

The following procedure is applicable to samples containing up to 400 mg of glucose, although a smaller sample should be taken if the citrate concentration permits. The neutral sample is placed in a 60 cc separatory funnel, the volume is brought to 15 cc, and 4.3 cc of 27 N sulfuric acid are added, or if a preliminary bromination has been made 15 or 20 cc of the filtrate are used.

2 cc of bromide-bromate and 1 cc of manganese sulfate are added, and the funnel is placed in an ice water bath. The manganese dioxide suspension is prepared as follows: 7.5 cc of 2 M manganese sulfate are placed in a small beaker immersed in an ice bath, or, better, a freezing mixture, and 4 cc of 27 N sulfuric acid are added. When the solution is below  $5^{\circ}$ , 7.5 cc of cold 26.1 per cent  $\text{NaMnO}_4 \cdot 3\text{H}_2\text{O}$  (1.33 M) are added with stirring. The solution should be allowed to stand in the bath for some minutes before use. The requisite (not total) amount of suspension is added to the funnel and allowed to stand for at least 3 hours at  $0^{\circ}$ . The funnel is inspected occasionally to see that the manganese dioxide is present in excess and that it has not settled out. At the end of this time the contents of the funnel are allowed to come to  $20-25^{\circ}$  and to stand for another hour. Then hydrazine is added and the analysis completed as usual.<sup>2</sup>

In the presence of smaller amounts of glucose (e.g., 50 mg) a suspension may be made from the 0.2 M potassium permanganate with 1 cc of manganese sulfate, 6.6 cc of 0.2 M  $\text{KMnO}_4$ , and 2 cc of 27 N sulfuric acid. This suspension may be used routinely for milk, animal urines, and in other cases in which the consumption of 0.2 M  $\text{KMnO}_4$  tends to exceed about 3 cc, whether because of glucose or other reducing substances.

<sup>2</sup> Since the water content of commercial sodium permanganate is variable, it is desirable to test how well the solution matches the manganese sulfate solution by centrifuging suspensions made with volumes varying slightly from the theoretical one specified and finding by inspection of the supernatant the proper volume to be used in subsequent determinations.

*Acetone Bodies*—Acetoacetic acid is removed by the preliminary bromination in addition to which Pucher, Sherman, and Vickery advise prior boiling of the acidified solution for 10 minutes. Krebs and Johnson (14) state that 1 hour's boiling eliminates the slight interference caused by the presence of oxalacetic acid. Interference from hydroxybutyric acid, we find, is due to its slow rate of bromination, with the main portion carried over into the second bromination. Since with this substance as well as with acetone, and especially in the dilutions used in tissue analysis, bromination products remain partly in solution, we find it advisable to extract them with petroleum ether before the second bromination.

Samples known to contain acetone or acetoacetic acid should be boiled undiluted in the presence of acid, restored to the original volume, and then used in the usual way.

### *Comment*

Because bacterial contamination can lead to the loss of much of the citrate in a few hours, it is essential that the urine be collected with an efficient preservative, especially 24 hour specimens and those from metabolism cages.

The preliminary treatment with bromine is necessary to remove small amounts of various urinary constituents which precipitate with bromine. Silver nitrate is added to remove unknown substances which cause the formation of stable emulsions with petroleum ether. Filtrates from the silver treatment always separate sharply after shaking is stopped. In the analysis of pure citrate solutions not more than 1.5 cc. of silver nitrate is added; an excess would cause precipitation of silver bromide in the next step. Urines usually contain sufficient chloride to react with several cc. of 2 N silver, hence an excess is improbable. The free bromine serves as an indicator, showing that silver is not present in excess.

Manual shaking of separatory funnels is time-consuming and uncertain. Warming by the hand necessitates occasional opening of the stop-cock, with attendant loss of solution. We have therefore constructed a simple shaking device which has been used with low boiling petroleum ether without mishap even at a room temperature of 35°. A motor of 1/50 horse power, adjustable to a speed of about 900 r.p.m., is used. An ordinary nail bent about 0.5 cm. is inserted into the chuck of the motor. (With a straight shaft the nail is wired alongside.) First, the stem of the funnel is inserted into a firmly clamped rubber stopper. Second, a string or wire, adequate to withstand sustained tension and friction, is placed around the neck of the funnel and attached to the eccentrically arranged nail. Third, a strong rubber band is placed around the neck of the funnel and attached to a fixed clamp in a direction opposite to the string attached

to the nail on the motor Alternate stretching and releasing of the rubber band agitates the suspension in the separatory funnel The stems of the funnels are cut off and ground down to an acute angle The over-all length of all funnels of each size should be the same to avoid shifting the clamp holding the rubber stopper No lubricant is used on the stopper of the 125 cc funnel It is wetted with a drop of the filtrate and inserted firmly with a slight, twisting motion A drop of water is applied to the stopper of the smaller funnel before it is shaken Stop-cocks should have the thinnest possible film of a heavy rubber paraffin grease When the funnel is removed from the shaker, the stop cock is always opened before the stopper to equalize the pressure

Many workers have used permanganate in large excess However, especially with small amounts of citric acid (less than 3 mg), it is important to add permanganate only as rapidly as it is used up Use of the bromide-bromate mixture, instead of bromide alone, reduces the amount of permanganate needed and makes for greater convenience and accuracy Since permanganate vigorously decomposes the intermediate acetone-dicarboxylic acid, its rapid reduction to manganese dioxide (hastened by the large excess of manganese sulfate) is obviously advantageous

Sufficient hydrazine must be added to remove the last trace of bromine, to avoid its extraction by the petroleum ether with which it would subsequently react Some of the bromine might later be reduced by the sulfite, giving rise to a high result We have avoided the use of ferrous sulfate for decolorizing because the color of the resulting ferric salt is indistinguishable from that of the free bromine Methods in which pentabromoacetone is filtered off result in a loss equivalent to about 3 mg of citric acid per 100 cc of reaction mixture, or an error of perhaps 50 per cent in urine of low citrate content In the petroleum ether procedure, the loss of pentabromoacetone has been shown to be negligible (about 0.001)

During the treatment with sulfite a trace of thiosulfate is formed, which, in the presence of silver halide, does not give the usual white precipitate turning to black with silver nitrate, but behaves like a halide and uses up an exactly equivalent amount of silver solution The thiosulfate formed is equivalent to about 0.03 cc of 0.4 N  $\text{AgNO}_3$  The treatment with hot ferric alum suffices to oxidize most of it, but for accurate results with small amounts it is desirable to run a blank, starting at the point in the procedure where the sulfite is added to the alcohol In our experience this amounts to only 0.01 cc

The principle of the titration is due to Kolthoff (15), but we have modified the details to our needs The thiocyanate solution, unlike that in the usual Volhard titration, need not be accurately prepared and need be only roughly pipetted The addition of a measured number of drops

is permissible, since an error of even 20 per cent in its measurement would result in an error of only 0.01 cc of silver nitrate. If the end-point is overstepped, additional thiocyanate may be added and the titration resumed.

*Mechanism*—The oxidation-bromination procedure used by us differs from that originally described by Kunz and used by succeeding workers. In the Kunz method, citric acid is treated with sulfuric acid and potassium bromide, then permanganate is added. We tried to carry out the reaction with a bromide-bromate mixture and were perplexed to find that the permanganate was not reduced and no pentabromoacetone was formed. The manganous salt essential for the reaction had been supplied by previous workers unwittingly when the potassium bromide used reduced the first portions of the permanganate. The reaction is autocatalytic and a trace of any easily oxidizable substance may serve as the trigger. It is probable that manganese dioxide is the ultimate oxidant, since the reaction proceeds smoothly if a suspension of this substance is added to an acidified citrate solution containing free bromine.

The course of the reaction as carried out by Kunz may be summarized as follows: (1) The permanganate first oxidizes bromide to bromine and is itself reduced to the manganous state. As this reaction approaches completion, and not until then, (2) the manganous salt thus formed reduces the next increment of permanganate to manganese dioxide. (3) The manganese dioxide then oxidizes citric acid to acetonedicarboxylic acid and (4) the latter is brominated stepwise with decarboxylation in the early stages. Step (1) has been demonstrated by interrupting the addition of permanganate just before the amount required by the bromide alone was reached. It was thus found that an insignificant amount of pentabromoacetone had been formed. Therefore, statements to the effect that acetonedicarboxylic acid and bromine are formed "simultaneously" are to be regarded as meaning taking place during the same physical operation.

Steps (3) and (4) have been verified by comparing the bromination rate of pure acetonedicarboxylic acid with the over-all rate of steps (1) to (4). It was found that the over-all rate is, within the limits of the error of the measurement, the same as the bromination alone, indicating that the oxidation to acetonedicarboxylic acid is very rapid and that the bromination is the rate-determining reaction. The speed of the total reaction at 18°, 24°, and 30° is described by the curves in Fig. 1.

*Acidity*—The original Kunz procedure gave results about 10 per cent lower than required by theory. Further work by others, with various changes in the bromination conditions, failed to increase the yield, so that the conversion to pentabromoacetone seems to be accepted as an

inherently "incomplete" reaction. We were therefore surprised to find that the apparent yield could be varied at will. In fact, an apparent yield of 105 per cent could be attained as easily as one of 75 per cent. The main factors favoring a high yield were high sulfuric acid and high citrate concentrations. There is no suggestion in the literature that any bromination product other than pentabromoacetone might be formed, yet the values above 100 per cent suggested that formation of some hexabromoacetone had occurred. This was verified by isolation, from a strongly acid bromination mixture, of a white crystalline product having the theoretical bromine content and melting at  $106^{\circ}$ . The melting point of hexabromoacetone is given by Weidel and Gruber (16) as  $107-109^{\circ}$ . On the other hand, by the use of a weakly acid mixture an uncrystallizable

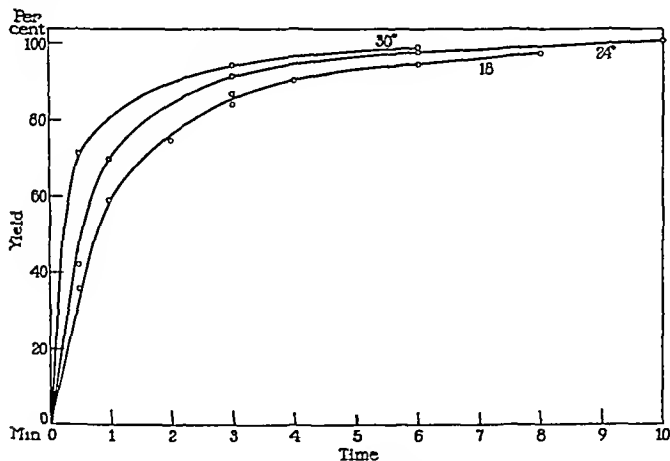


Fig 1 Rate of bromination of citric acid at  $18^{\circ}$ ,  $24^{\circ}$ , and  $30^{\circ}$

oil was obtained which averaged about 3.7 atoms of bromine per mole, and consisted essentially of a mixture of tetra- and tribromoacetones. The recovery with varying acidity is shown in Fig 2.

These results indicate that in any so called pentabromoacetone method a mixture of bromoacetones occurs, the proportion of each depending on several factors, the most important of which is the sulfuric acid concentration. Thus the bromination product approximates a tetrabromoacetone rather than a pentabromoacetone, when, as in one method, the final sulfuric acid concentration is 1 N and the citrate 1 mg or less. Fig 2 shows that above 9 N sulfuric acid the yield falls off, even though in this region hexabromoacetone replaces some of the expected pentabromoacetone. Here the strong acid by its higher oxidative potential may

induce scission of the carbon chain. At 15 N, when pure hexabromoacetone is obtained, the apparent yield is only 72 per cent, or 60 per cent in terms of moles of hexabromoacetone. This indicates destruction of 40 per cent of the citrate.

*Temperature*—Kunz, followed by others, prescribed a temperature of 50° for the bromination, while some advised room temperature. Fasold stated that at 55° results were 30 per cent too low. Reichard advised cooling to 5° or less. Our work shows a slight decrease in yield (1 per cent) from 24° to 48° and a 1 per cent increase at 0°. Qualitatively this is to be expected in the exothermic bromination reaction, but the quantitative effect of temperature variation can be calculated from van't

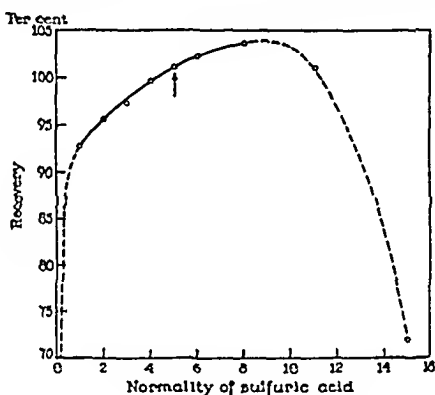


FIG. 2 Recovery with varying acidity

Hoff's equation. These calculations are in good agreement with our findings where they have been checked.<sup>3</sup>

<sup>3</sup> We select, for example, the point at which 101 per cent recovery is obtained (about 10 mg) and find that this corresponds to 1 mole of hexabromoacetone to 19 moles of pentabromoacetone. The equilibrium constant for the reaction pentabromoacetone + Br<sub>2</sub> → hexabromoacetone + HBr may then be written  $K_4 = (\text{hexabromoacetone})/(\text{pentabromoacetone}) = 1/19$ . Since we solve for the ratio of the constants at two different temperatures, and since practical constancy of the Br and HBr concentrations is assured by the experimental conditions, these concentrations need not be expressed. From the data of Magee and Daniels (17) we find values of  $\Delta H = 6.5, 15, 12$ , and 8 kilocalories for the bromination of an aliphatic hydrogen in methane, and mono-, di-, and triphenylmethane respectively. We can assume a rounded average of 10 kilocalories for the bromination of the residual hydrogen of pentabromoacetone. This may seem rash, but calculation shows that either doubling or halving the value does not alter the outcome materially. Substituting in the integrated form of the van't Hoff relation, we have for 48° and 24°,  $\log K_{48} - \log K_{24} = -10,000/4.57 (1/297 - 1/321)$ , or  $K_{48}/K_{24} = 0.288$ , a yield of 100.3 per cent, i.e.,<sup>3</sup>

*Citrate Concentration*—Fig 3 shows the effect of the citrate sample on the yield. The yield is seen to be strictly stoichiometric only with a 3 mg sample. With another acidity or temperature the value would be different. Nevertheless, exact results may be obtained by employing a curve con-

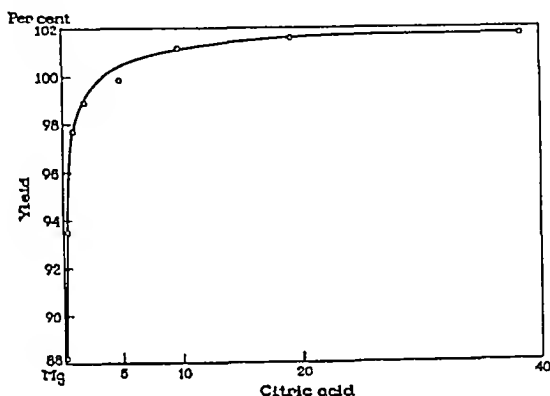


Fig 3 Recovery with different concentrations of citric acid

structed from the empirical factors given in Table I. For most work the factor 1.53 may be used routinely without serious error.<sup>4</sup>

diminution of 0.7 per cent as a result of a 24° rise in temperature. Interestingly this value of 0.7 per cent is not constant, but would vary with the initial deviation from 100 per cent. From 102 per cent the value would fall to 100.6 per cent (1.4 per cent). Similar computation in the 1 and 2 mg region (about 98 and 99 per cent recovery) shows a larger effect. Assuming the same heat of bromination for tetrabromoacetone → pentabromoacetone as for pentabromoacetone → hexabromoacetone, we find decreases of 5.7 and 2.2 per cent respectively, at 48° compared to 24°. One important factor is not taken into account in this theoretical analysis, namely, the effect of an elevated temperature on the clastic action of the permanganate. With pure citrate solutions, and provided the addition of permanganate is carried out as advised, loss from increased clastic action may be considered negligible, but in the presence of considerable amounts of glucose even room temperature is too high and it is necessary to work at 0°. Glucose, if present in large amounts, must be burned completely to CO<sub>2</sub> and water, which increases considerably the amount of oxidant used. In this case, as described before, permanganate should be replaced by manganese dioxide.

<sup>4</sup> The rapid change of slope of the curve (Fig 3) at the lower values is associated with the threshold of solubility of the pentabromoacetone. All of the bromoacetone from 0.6 mg of citric acid is in solution, while that in excess exists in a second liquid phase consisting of pentabromoacetone with a small proportion of hexabromoacetone and whatever bromine is dissolved. In the aqueous phase, however, tetrabromoacetone and pentabromoacetone coexist in a ratio to give an apparent yield of about 96 per cent. Since the proportion of hexabromoacetone in the emulsoid phase would



Fig 3 demonstrates the necessity of keeping at a minimum the dilution which results from the use of many reagents. We use sulfuric acid and other reagents in fairly concentrated solutions. The unnecessarily large and variable volumes of permanganate in use are undesirable for the reason just stated and because the consequent variations in acidity cause variations in yield, as shown in Fig 2.

*Precision*—The factors in Table I are based on multiple determinations made on 40, 20, 10, 5, 2, 1, 0.4, and 0.2 mg of citric acid samples. The average deviation from the mean in the group from 40 to 5 mg was less than 0.2 per cent, the 2 and 1 mg samples showed an average deviation less than 0.4 per cent. The 0.4 and 0.2 mg determinations were fortu-

TABLE I  
*Empirical Factors (Theoretical Factor = 1.537)*

0.4 N AgNO <sub>3</sub>	Factor	0.4 N AgNO <sub>3</sub>	Factor
cc		cc	
0.10	1.757*	2.00	1.539
0.15	1.695*	3.00	1.531
0.20	1.660*	4.00	1.527
0.30	1.622*	5.00	1.525
0.40	1.601*	7.00	1.520
0.50	1.586*	10.00	1.517
0.70	1.570	15.00	1.514
1.00	1.557	20.00	1.512
1.50	1.545	25.00	1.510

\* These factors are for amounts below the recommended range. The fourth place is supplied to enable a smoother curve to be drawn, if desired.

*Computation*—The number of cc of 0.4 N AgNO<sub>3</sub> consumed, multiplied by the factor appropriate for it, equals the mg of anhydrous citric acid in the aliquot subjected to oxidation. Subtract the 0.05 cc used up by the thiocyanate and whatever blank value is found (e.g. 0.01 or 0.02 cc) from the burette reading to find the cc of 0.4 N AgNO<sub>3</sub> consumed.

tously precise, but errors of several per cent are to be expected at this level. The determinations from 1 to 40 mg were repeated independently by H. H. Taussky. The average difference between our results for each sized sample was 0.4 per cent.

Table II shows the recovery of citrate added to human urine. Urine A

be constant regardless of the amount of citrate taken, the analytically determined yield must be the weighted average of the organic bromine in the two phases. In other words, the composition of the emulsoid phase (the value the upper limb of the curve approaches asymptotically) and the composition of the aqueous phase (determined by analysis of a 0.6 mg sample) determine the curve at all intermediate points.

was from a patient who had been "acidified" with four 1 gm doses of ammonium chloride daily and therefore excreted a diminished amount, 24 mg, of citric acid in 24 hours. Urine B was a 24 hour specimen from a presumably normal subject and consisted of 815 cc containing 599 mg of citric acid.

As our standard we used a well crystallized sample of potassium citrate monohydrate. After exposure of the sample for weeks there was no appreciable gain or loss on periodic weighing. The potassium content was determined by conversion to the sulfate (Calculated, K 36.16 per cent, found, 36.03 per cent). As an additional check these determinations were repeated on another single, large crystal of potassium citrate from another source with comparable results.

Several different agents have been employed to liberate the bromine in the pentabromoacetone from its organic combination and make it accessible to titration. The reagent we use, neutral sodium sulfite, was found by

TABLE II  
*Recovery of Citric Acid Added to Urine*

Urine	Citric acid in native urine	Citric acid added	Total citric acid	Found	Per cent of total
		mg	mg	mg	
A	0.186 mg per 12.5 cc	2.402	2.588	2.570	99.3
	0.186 " " 12.5 "	2.402	2.588	2.562	99.0
	0.186 " " 12.5 "	7.685	7.871	7.828	99.4
	0.149 " " 10 cc	6.148	6.297	6.260	99.4
B	7.346 " " 10 "	7.205	14.551	14.405	99.0

accident to decompose a pentabromoacetone precipitate. Further investigation showed that the reaction could be made quantitative and that it is as precise and dependable as the titration of inorganic bromide itself. It is rapid, being 50 per cent complete in a few seconds at room temperature.

The sulfite reagent should have a pH of about 7.6. At higher values bromoform and dibromoacetic acid would be formed, at lower values there would be a loss of sulfur dioxide.

The use of this mild sulfite reagent, which probably depends for its action on formation of an intermediate with the ketone, offers additional assurance of specificity to the already highly specific Cahours-Stahre reaction.

#### SUMMARY

1. A precise method for the determination of citric acid in pure solution and in urine is described. The range of the method is from 1 to 40

mg, with a precision of 0.5 per cent. With suitable care even 0.2 per cent is attainable above 5 mg.

2 The "pentabromoacetone reaction" has been studied and it is shown that tetrabromoacetone and hexabromoacetone are also found. The conditions governing the relative amounts of each bromoacetone formed are discussed. The main factors are acidity, citrate concentration, and temperature.

3 The reaction mechanism has been clarified, leading to the introduction of new reagents with certain advantages. The decomposition of pentabromoacetone is affected by a new, mild reagent which permits a precise, direct titration with silver nitrate.

We wish to express our thanks to Hertha H. Taussky for her help and many courtesies and also to Dr. Harry Sobotka for his valuable advice.

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# THE AMINO ACID COMPOSITION OF HUMAN MILK PROTEINS

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It has generally been considered that human milk is of superior nutritional value for infants as compared with cow's milk. This has been based on clinical observations since comparatively little is known of the composition of human milk and especially of its proteins. The investigation of the amino acid constituents of the cow's milk proteins has long held the attention of many workers. However, in the case of the proteins of human milk, few and very incomplete amino acid analyses have been reported (1-3).

The complete analysis of the proteins from human milk would make for a better understanding of human nutrition, since these proteins are utilized at a period of life when the rate of growth is at its maximum. Although other factors are of importance in determining the "biological value" of proteins, the amino acid composition remains the leading factor in the evaluation of this nutritional concept. The analysis of human milk proteins has been undertaken with these considerations in mind.

## EXPERIMENTAL

The human milk used in this investigation represented approximately 500 collections from both white and Negro women. As the samples were collected, they were stored in a common container and kept frozen until a sufficient quantity had been accumulated. The human milk proteins were isolated and purified according to the method to be described. It was considered to be of value to analyze similar proteins at the same time to serve as a check on the results and as a basis of comparison with the human milk proteins. The proteins of cow's milk were used for this purpose. Cow casein was obtained by isoelectric precipitation. The lactoglobulin was removed from the cow's milk whey by half saturation with magnesium sulfate before the isolation of the lactalbumin (4, 5).

The moisture, ash, and nitrogen content (micro-Kjeldahl) of each protein was determined. On an ash- and moisture-free basis the nitrogen content of the proteins was found to be cow casein, 15.28 per cent, cow lactalbumin, 14.92 per cent, human casein, 14.90 per cent, human lactalbumin, 14.94 per cent. The results reported in this paper are all corrected to a moisture- and ash-free basis.

*Preparation of Human Casein*—5 gallons of composite breast skim milk

were warmed to 35–38° and the acidity adjusted to pH 4.5 by the slow addition of 0.1 N acetic acid with vigorous stirring. The milk was then centrifuged and the casein collected. The casein was suspended in sufficient water to make a 1 per cent solution and dissolved by the slow addition of 0.05 N NaOH to pH 6.2. After filtration, the casein was again precipitated and the procedure repeated three times more. The solution of casein was finally dialyzed under toluene against running tap water for 72 hours and then against 10 to 20 volumes of distilled water for 24 hours more.

After dialysis, the casein solution was adjusted to pH 4.5 and centrifuged. The casein was washed twice with alcohol and ether and dried over sulfuric acid *in vacuo*.

*Preparation of Human Lactalbumin*—The supernatant whey after the removal of the casein was adjusted to pH 5.0. It was then slowly heated to 80° in a water bath and held at this temperature for 30 to 45 minutes. The suspension of coagulated protein was allowed to cool and settle and the supernatant liquid was removed. The protein was centrifuged, suspended in water, and 0.05 N NaOH added to pH 6.5. After filtration, the protein was precipitated with acetic acid (pH 4.8 to 4.9) and centrifuged. The process was repeated three times. The solution of protein was finally dialyzed against tap and then distilled water, precipitated as before, and centrifuged. It was washed twice with alcohol and ether and dried *in vacuo* over sulfuric acid.

The lactalbumin preparation was expected to contain the other coagulable whey proteins. However, these are present in relatively insignificant amounts (6).

*Amino Acid Analyses*—The results of the analysis and the source of the method used for each amino acid are shown in Table I. Also shown is the per cent of the total nitrogen contributed by each amino acid to indicate the actual amount of the protein which has been accounted for. In order to check the results, there is included a comparison with the values reported by previous authors. The results, in general, are in reasonable agreement with those obtained by similar methods of analysis.

In the analyses which were determined by colorimetric methods (tyrosine, glycine, proline, cystine, arginine, phenylalanine, methionine, and tryptophane) purified synthetic amino acids dried over phosphorus pentoxide were used as standards. Those methods which depend on the formation of a volatile substance from the amino acid being tested (alanine, serine, leucine, isoleucine, threonine, and valine) were checked by testing the procedure with purified synthetic amino acids. All the color comparisons were made in a Beckman spectrophotometer and the wave-length of maximum absorption was determined, unless previously described in the literature.

TABLE I  
Amino Acid Composition of Human and Cow's Milk Proteins

Amino acid (method of analysis)	Cow casein			Human casein			Cow lactalbumin			Human lactalbumin		
	Reported in literature		Per cent of total N	Reported in literature		Per cent of total N	Reported in literature		Per cent of total N	Reported in literature		Per cent of total N
	per cent	per cent		per cent	per cent		per cent	per cent		per cent	per cent	
Tyrosine (7)	5.5	2.8	5.2 (8), 5.8 (2), 5.0 (3)	5.5	2.9	5.5 (2), 6.1 (3)	3.5	1.8	3.4 (5), 3.6 (1)	4.5	2.3	4.5 (2), 5.2 (3)
Alanine (8)	2.3	2.4	2.7 (9), 1.5 (10)	2.0	2.1		2.6	2.8	2.4 (11)	2.5	2.6	
Glycine (12)	0.4	0.5	0.5 (12)	0	0		0	0	0 (12), 0.4 (11)	0	0	
Proline (13)	8.1	6.5	7.6 (24), 7.0 (13)	8.9	7.3		4.0	3.3	3.8 (11)	3.5	2.8	
Glutamic acid (14)	21.9	13.7	22.0 (14, 25)	20.9	13.4		13.7	8.7	12.9 (11)	12.5	8.0	
Aspartic " (14)	4.2	2.9	4.1 (9)	4.6	3.3		9.6	6.8	9.3 (11)	9.3	6.6	
Serine (15)	5.0	4.4	2.3 (9), 5.0 (15)	5.4	4.8		4.0	3.6	1.8 (11), 4.3 (15)	1.2	3.7	
Cystine (16)	0.4	0.3	0.4 (1), 0.3 (3, 8)	0.6	0.5	0.6 (1), 0.7 (3)	3.1	2.4	3.1 (8), 3.3 (1)	3.8	3.0	4.4 (1), 3.1 (3)
Arginine (17)	3.9	8.2	3.7 (1), 3.8 (17)	3.4	7.4	3.6 (1), 3.3 (3)	3.6	7.7	3.0 (8), 4.0 (1)	5.0	10.8	5.0 (1), 5.2 (3)
Phenylalanine (18)	5.5	3.1	5.8 (8), 5.0 (18)	5.8	3.3		4.5	2.6	4.8 (8)	4.8	2.7	
Leucine (19)	14.4	10.1	14.8 (8), 12.1 (19)	12.2	8.7		17.4	12.4	17.2 (8), 14.3 (11)	16.7	11.9	
Isoleucine (19)	5.2	3.6	5.1 (8)	6.3	4.6		4.2	3.0	4.2 (8)	4.3	3.1	
Histidine (20)	2.0	3.6	1.9 (8), 1.7 (1), 1.8 (3)	2.0	3.6	1.5 (1), 1.8 (3)	1.4	2.6	1.8 (1), 1.4 (3, 8)	1.5	2.8	1.7 (1), 1.2 (3)
Lysine (21)	6.0	7.5	5.9 (3), 6.1 (1), 5.8 (26)	5.6	7.2	5.5 (1), 5.2 (3)	6.2	8.0	6.3 (1), 5.2 (3), 5.9 (8)	6.6	8.5	6.6 (1), 5.8 (3)
Threonine (22)	4.6	3.5	4.6 (8), 4.4 (22)	4.5	3.5		4.3	3.4	4.5 (8)	4.0	3.1	
Methionine (23)	3.1	1.9	2.9 (1), 3.0 (8, 23)	2.3	1.5	2.7 (1), 2.2 (3)	2.4	1.5	2.3 (1), 2.2 (3)	1.7	1.1	1.4 (1), 1.9 (3)
Tryptophane (7)	1.3	1.2	1.4 (2), 1.1 (3), 1.2 (8)	1.5	1.4	1.1 (1), 1.7 (3)	2.1	1.9	2.0 (8), 1.9 (2), 1.8 (3)	2.3	2.1	2.5 (2), 2.3 (3)
Valine (19)	5.3	4.2	5.2 (8), 7.0 (19)	5.0	4.0		4.0	3.2	4.0 (8)	4.1	3.3	
Total	99.1	80.4		96.5	79.5		90.6	75.7		91.3	78.4	

## DISCUSSION

The data obtained in this investigation contribute to a better understanding of infant nutrition. It has been the practice to use cow's milk diluted to about 50 per cent of its original protein content when human milk is not available for young infants. As shown in Table II, there are differences between the total amino acids in human milk and those in diluted cow's milk. This table shows the amino acid content, in mg per 100 cc, of cow's milk, human milk, and cow's milk diluted to 50 per cent of its protein content. Although the total protein content as well as the proportion of

TABLE II

*Comparison of Amino Acid Content of Cow's Milk and Human Milk from Their Protein Analyses*

Figures are mg per 100 cc

Amino acid	Cow's milk 2.8 per cent casein, 0.5 per cent lactalbumin	Human milk 0.5 per cent casein, 1.0 per cent lactalbumin	Diluted cow's milk 1.4 per cent casein, 0.25 per cent lactalbumin
Tyrosine	172	73	86
Alanine	75	35	37
Glycine	11	0	6
	250	80	125
Glutamic acid	680	230	340
Aspartic "	166	116	83
Serine	160	69	80
Cystine	27	41	14
Arginine	127	67	64
Phenylalanine	177	77	88
Leucine	490	228	245
Isoleucine	167	75	83
Histidine	63	25	32
Lysine	200	94	100
Threonine	151	63	76
Methionine	99	29	50
Tryptophane	47	31	24
Valine	171	66	86

casein to lactalbumin varies in milk, the average composition is reported to be approximately 2.8 per cent casein, 0.5 per cent lactalbumin in cow's milk, and 0.5 per cent casein, 1.0 per cent lactalbumin in human milk (5, 6, 27, 28).

It is expected that cow's milk should contain larger total quantities of all of the amino acids than human milk since the protein content is twice as great. This holds true with the exception of cystine. However, in the group of essential amino acids there are noteworthy differences between

human milk and the diluted cow's milk. Here, the human milk contains 3 times as much cystine as does the diluted cow's milk. On the other hand, the diluted cow's milk proteins contribute over  $1\frac{1}{2}$  times as much methionine to the milk as do the human milk proteins. In the case of the human milk, the lower methionine content probably does not present a nutritionally critical condition since the larger amount of cystine may spare the requirement of methionine (29). To put the comparison on a quantitative basis, the proteins in 100 cc of human milk contain 0.36 mM of sulfur amino acids (cystine = 0.17, methionine = 0.19), while the diluted cow's milk proteins contain 0.38 mM (cystine = 0.05, methionine = 0.33).

Another important difference is the higher content of tryptophane in human milk as compared to the diluted cow's milk. Of the other essential amino acids, there are to be noted differences of about 20 per cent in the case of histidine, threonine, and valine, the human milk being lower. However, it has been shown that histidine is not required by adults to maintain nitrogen equilibrium over an experimental period of about a week (30).

#### SUMMARY

1 The casein and lactalbumin from human and cow's milk were isolated and the amino acid content for eighteen amino acids determined.

2 In a comparison of human milk and cow's milk diluted to 2 volumes, the analyses indicate that there are over 3 times as much cystine in the human milk. However, there was less methionine found in the human milk. On the basis of millimoles of total sulfur amino acids, there is no significant difference between the two.

3 The diluted cow's milk was shown to contain higher percentages of valine, threonine, and histidine while human milk was found richer in tryptophane.

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# A SIMPLE MODIFICATION OF THE COLORIMETRIC METHOD FOR ROUTINE THIAMINE CLEARANCE TESTS

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The accurate diagnosis of latent thiamine deficiency in man is difficult even for clinicians specializing in vitamin nutrition (1). Accordingly, attempts have been made to develop simple and reliable objective tests for thiamine deficiency (2). All these tests depend upon the measurement of the urinary excretion of the vitamin under standardized conditions.

The most specific of the procedures for the determination of thiamine in urine is the colorimetric method of Melnick and Field (3) based upon the reaction of the vitamin with diazotized *p*-aminoacetophenone. This procedure requires no blank test for ascertaining true thiamine content. To improve the specificity of the thiochrome method of Hennessy and Cerecedo (4, 5) a sulfite blank (6) has been incorporated to correct for the presence of variable amounts of interfering fluorescent compounds, or a correction has been included based upon the determination of one of the more important interfering substances (7). Another modification, involving photometric measurement of light absorption of thiochrome at 365 m $\mu$ , rather than its fluorescence, has appeared recently (8). Here correction is made for irrelevant absorbing materials by a procedure involving inhibition of the conversion of thiamine to thiochrome by treatment with benzenesulfonyl chloride. Likewise, the fermentation method of Schultz, Atkin, and Frey (9) has undergone modifications in attempts to correct for the high concentrations of other yeast-stimulating substances in urine (10-14). The thiochrome and fermentation methods involve use of specialized apparatus, and the necessary modifications have added to the complexity of the tests. The original colorimetric method for the determination of thiamine in urine (3) also involved specialized apparatus, making the procedure of value only in research studies and for routine thiamine clearance tests in the clinical laboratory.

In the present report a simple and rapid modification of the colorimetric method is presented which lends itself to routine use as a means for determining thiamine status of patients. By the simple expedient of testing a 1 hour urine aliquot, it has been possible to eliminate the benzyl alcohol extraction step for the quantitative and selective removal of thiamine from the concentrated urine. There are insufficient salt (15) and other materials (16) in a 1 hour urine sample to interfere with the adsorption of

thiamine on the zeolite column. The precaution of maintaining an inert atmosphere during the adsorption and elution of the vitamin has been found unnecessary, this allows simplification of the apparatus and technique for removing the thiamine from compounds in urine which interfere with the coupling reaction. The color development has been modified slightly to conform with the procedure of the United States Pharmacopoeia (17). The acidification-alkalization, prior to the removal of the xylene solution containing the azo dye, has been dispensed with, since the increase in precision resulting from this step is not warranted in determining urinary thiamine for diagnostic purposes. By the simplified procedure twelve to fifteen samples of urine may be analyzed by a single analyst in an 8 hour day, using only such apparatus as is readily available in the routine clinical laboratory.

### Procedure

**Reagents—Zeolite**<sup>1</sup> Approximately 50 mesh. This is first prepared in bulk by stirring with four 10 volume portions of 3 per cent acetic acid for 10 minutes each. Between the second and third acid wash, a 15 minute treatment with 5 volumes of 25 per cent potassium chloride is introduced. The zeolite is washed thoroughly with water, alcohol, and ether, dried in air, and stored in a sealed bottle.

**Salt solution** 25 per cent potassium chloride in 0.1 N hydrochloric acid.

**p-Aminoacetophenone solution** 6.35 gm of p-aminoacetophenone are dissolved in 90 cc of concentrated hydrochloric acid, and diluted to 1000 cc with distilled water. The solution is stable for at least 6 months if stored in an amber bottle and protected from direct sunlight.

**Sodium nitrite solution** 22.5 gm of sodium nitrite are dissolved in sufficient distilled water to make 500 cc. This solution is stored in the refrigerator and is stable for at least 3 months.

**Sodium hydroxide-sodium bicarbonate buffer** 40 gm of sodium hydroxide are dissolved in 1500 cc of distilled water, and 57.6 gm of sodium bicarbonate are added, followed by sufficient distilled water to bring the volume to 2000 cc.

**Diazotized p-aminoacetophenone solution** 5 cc of the p-aminoacetophenone solution are pipetted into a 50 cc graduate surrounded with chopped ice and provided with a stirrer. 5 cc of sodium nitrite solution are added slowly with constant mixing, and the solution gently stirred for 10 minutes. 20 cc of the nitrite solution are again added slowly, and

<sup>1</sup> Decalco, obtained from The Permutit Company, New York. Zeolite of the proper mesh should be used since adsorption and elution of the thiamine are a function of the area of the particles exposed to the solution.

the stirring continued for an additional 30 minutes. The temperature of diazotization should not exceed 5°. This solution should be used within 24 hours after preparation and should be kept below 5°.

**Thiamine reagent.** To 274 cc of the sodium hydroxide-sodium bicarbonate buffer, 20 cc of the diazotized *p*-aminoacetophenone solution are added rapidly with agitation. The reagent is ready for use when the initial faint purple coloration is replaced by a pale yellow. This usually occurs in 5 to 20 minutes. The reagent is prepared immediately before use. The quantities given are sufficient for ten samples plus a standard.

**Phenol-alcohol solution.** 15.6 gm of phenol are dissolved in sufficient 95 per cent alcohol to make 2000 cc. The solution is stored in an amber-colored bottle and is stable.

**Standard thiamine solution.** 50  $\gamma$  of anhydrous, U S P thiamine hydrochloride per cc of a 25 per cent alcohol solution at pH 1 to 2. This solution is stored in the refrigerator and is stable.

**Sodium hydroxide solution,** 2.0 N

**Brom-cresol green paper**

**Thymol blue indicator.** 1.0 gm of thymol blue is dissolved in 100 cc of 95 per cent alcohol.

**Xylene**

**Special Apparatus.**—The apparatus for the concentration of the urine sample and the removal of interfering substances is shown in Fig 1. A glass funnel is connected to a condenser by means of a short piece of rubber tubing. The latter is similarly joined to a tube having an internal diameter of 8 mm for a length of 4.5 inches, followed by a constricted portion about 4 mm in diameter. A plug of glass wool is placed at the top of the constriction, and exactly 3.0 gm of the treated zeolite added to the tube. The other features of the apparatus are clearly indicated in Fig 1. In the absence of a laboratory steam line, a 1 liter flask of boiling water, equipped with a 2-way stop-cock, may be employed.

**Adsorption and Elution of Thiamine.**—A 1 hour aliquot of the urine<sup>2</sup> is adjusted to pH 4.5, with brom-cresol green paper, and then passed through the zeolite column at room temperature. A filtration rate of 3 or 4 drops per second is easily maintained by the application of a mild suction, controlled by means of the 2-way stop-cock. Steam is then passed through the outside jacket and 30 cc of water are poured on the column. This is allowed to heat for  $\frac{1}{2}$  minute and then drawn rapidly through the zeolite with maximal suction. The thiamine is eluted immediately by passing 10 cc of the potassium chloride solution down the wall of the hot con-

The samples are collected in bottles containing 1 cc of 3.5 N sulfuric acid per 1 hour sample. Since no phosphorylated thiamine is excreted in urine (3), it is not necessary to treat the samples with a phosphatase.

denser The eluate is collected in the receiving tube at the rate of approximately 1 drop per 2 seconds, and the last few drops are drawn through by suction. The zeolite column is washed with 200 cc of distilled water under full suction and with steam passing through the jacket. The column is finally cooled to room temperature by running the last 50 cc

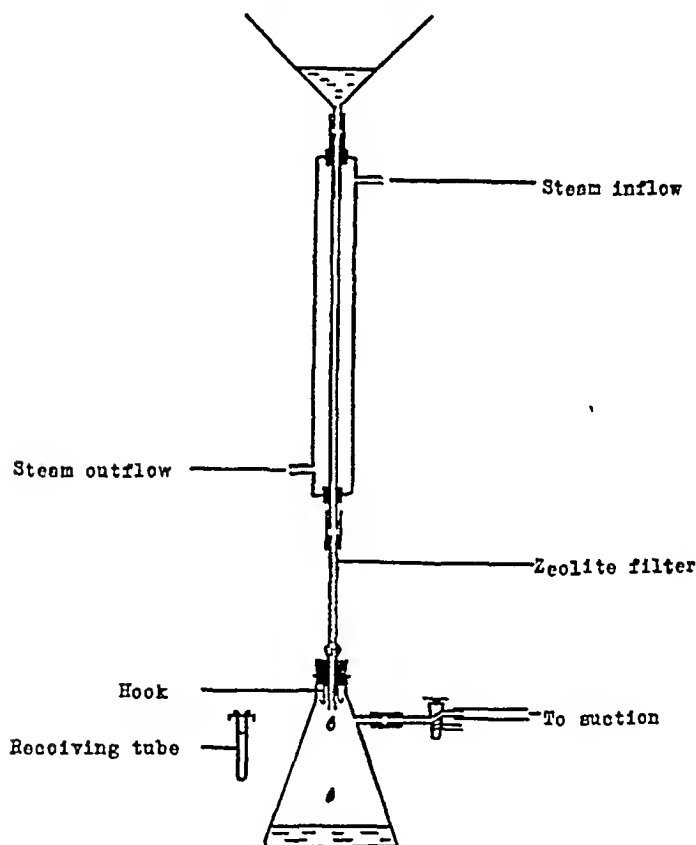


FIG 1 Simplified apparatus for adsorption and elution of thiamine in urine

of wash through with the steam turned off. The apparatus is then ready for the next sample. A standard thiamine solution containing 25  $\gamma$  in 50 cc of acidulated water at pH 4.5 is sent through the column in the same manner as the urine samples, preferably at the beginning of the series of tests.

*Color Development and Measurement*—The eluate is transferred to a 100

cc centrifuge tube 10 cc of the phenol-alcohol reagent (previously passed through the receiving tube as a wash) are added, and then 2 drops of thymol blue indicator. Before the next step is undertaken, all samples under test are brought to this stage and the color is developed in all simultaneously.

The sodium hydropoxide solution is added dropwise until the first distinct blue color is produced in the agitated solution. 25 cc of the thiamine reagent are added immediately. The solution is mixed (cork stoppers being used) and allowed to stand in the dark at room temperature for a period of 2 or more hours. 5 or 10 cc of xylene are then added, and the tube shaken vigorously for 3 minutes to extract the red pigment formed by the reaction between thiamine and the diazotized *p*-aminoacetophenone. The liquids are separated by centrifugation. The supernatant xylene layer is then transferred<sup>3</sup> to a Nessler tube, visual photometer cup, or the absorption cell of a photoelectric colorimeter, and compared with the standard solution.<sup>4</sup>

#### RESULTS AND DISCUSSION

Analyses were conducted on nineteen urine samples which were obtained from subjects excreting thiamine in the normal and deficient ranges, according to two types of clearance tests. These involved measurement of the 24 hour urinary thiamine following oral, postprandial dosage with 50 mg of thiamine (18, 1) and of the 4 hour thiamine excretions when fasting subjects in the postabsorptive state received 0.35 mg of thiamine per sq.m of body surface.<sup>5</sup> The results obtained by the original and critically tested Melnick-Field procedure (3) and those yielded by the simple modification, herein described, are presented in Table I.

In the present study, measurements of the red color were made in the Evelyn photoelectric colorimeter with the 520  $m\mu$  filter.<sup>6</sup> The agreement between the two methods is considered good, particularly for clinical purposes, in view of the wide differences in values of urinary thiamine excretion between normal and deficient subjects. It is primarily for the latter reason that the original fermentation (9, 10) and thiochrome procedures (4) have found acceptance in some clinical laboratories despite

<sup>3</sup> The transfer is readily effected by drawing off the xylene layer through a U tipped pipette attached to a rubber bulb.

<sup>4</sup> The red pigment is stable in xylene for many months. Though the intensity of the color for a given amount of thiamine depends on the freshness of the reagent, a permanent set of standards may be made up for use with a visual comparator block. These should be stored in the dark and sealed to prevent evaporation of the xylene. The error in this procedure is less than 10 per cent.

<sup>5</sup> Estimated from the height and weight of the subject (19).

<sup>6</sup> The instrument and filter are supplied by the Rubicon Company, Philadelphia.

the fact that the values reported as thiamine deviated more than 100 per cent (6, 11, 12) from the true figures

In an earlier paper (1) the merits and limitations of various thiamine clearance tests were discussed. Advantages were cited for measuring the urinary thiamine values following administration of a test dose of extra vitamin, particularly when the latter is given intramuscularly in small dosage. These clearance tests now have the further advantage of allowing use of the simple modification of the colorimetric assay procedure. Sufi

TABLE I

*Comparison of Results Obtained in Analysis of Urine by Original (8) and Simplified Colorimetric Procedures*

Subject No	Range of urinary thiamine	Total thiamine excretion after test dose			
		Oral*		Intramuscular†	
		Simple method	Original method (2)	Simple method	Original method (2)
		$\gamma$ per 24 hrs	$\gamma$ per 24 hrs	$\gamma$ per 4 hrs	$\gamma$ per 4 hrs
1	Normal	980	830	110	90
2	"	1630	1450	160	160
3	"	1080	1070	130	100
4	"	1620	1460	140	120
5	"	1360	1290		
6	Deficient	140	170	25	22
7	"	180	220	28	37
8	"	260	230	32	38
9	"	180	190	37	30
10	"	200	210	43	37

\* 5 mg of thiamine were taken orally after a large midday meal, and urines were collected during the subsequent 24 hour period

† 0.35 mg of thiamine per sq m of body area were injected intramuscularly 12 hours after the last meal, and the subsequent 4 hour urine samples collected from the fasting subjects

cient thiamine is excreted so that the limited sensitivity of the original method is no longer an obstacle to its use

Of the several techniques described in the literature for the diagnosis of thiamine deficiency, the 4 hour thiamine clearance test (1) is regarded as the most convenient and versatile (2). The present report may be regarded as a companion paper in that analytical difficulties operating against its routine use by the clinician have now been eliminated

#### SUMMARY

A simple adaptation of the Melnick-Field colorimetric method for the determination of thiamine in urine is presented. The procedure involves

direct adsorption of the vitamin from a 1 hour urine sample on a simplified zeolite column, its elution and coupling with diazotized *p*-aminoacetophenone, and finally extraction and measurement of the pigment formed. The results obtained by the abridged method in testing urine samples varying widely in thiamine content compared favorably with those yielded by the original procedure. Twelve to fifteen samples of urine can be analyzed by a single analyst in 8 hours of working time, using only such apparatus as is readily available in a routine clinical laboratory.

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# DETERMINATION OF CARBON MONOXIDE IN GAS MIXTURES\*

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The advantages of using blood as the active reagent in the determination of carbon monoxide in gas mixtures have been considered in a recent evaluation of principles and methods of CO analysis (Sendroy (7)). The foremost advantage lies in the strong affinity, and, in the absence of oxygen the specificity, of hemoglobin for CO, and its indifference to the presence of certain non-atmospheric gases which are frequently present and which usually must be removed in other methods of CO analysis because they react with the reagents used. This well known and previously used principle is most accurately and conveniently applied to the analysis of gas mixtures containing CO in low concentrations, when the Van Slyke-Neill (13) gas apparatus is used for (a) the absorption and isolation of CO from the rest of the original gas mixture, and (b) its subsequent release and measurement under the optimum conditions afforded by that adaptable instrument. A method embodying the foregoing principle and technique, described by Sendroy (5), has been modified and improved in this laboratory. The increasing importance, for military and industrial operations, of studies of the physiological and pathological effects of the contamination of air by CO makes desirable the description of this revised method.

The following procedure for the determination of CO in air and other gas mixtures, in concentrations of from 0.05 to 0.8 volume per cent, employs suitable modifications of the absorption of CO with blood as previously carried out (5), and of the subsequent measurement of the blood CO content by what is essentially the technique of Van Slyke and Hiller (11) for CO capacity as a measure of blood total hemoglobin. The modification of the latter method and its application to the measurement of CO content were stimulated by improvements in CO blood analyses recently developed in Roughton's (1-3) and Van Slyke's (12) laboratories. The results of the several changes in technique are a saving of time and labor, and a reduction of the margin of error of Sendroy's (5) original method to  $\pm 1.2$  per cent.

\* The content of this paper is taken in part from a thesis submitted by Edward J. Fitzsimons in partial fulfillment of the requirements for the degree of Master of Science in the Graduate School, Loyola University.

### Reagents

*Sodium hyposulfite solution* 15 gm of  $\text{Na}_2\text{S}_2\text{O}_4$  are dissolved in 100 cc of 2 N NaOH. This solution is prepared fresh daily, and kept under oil ((13) p 534). It serves both for absorption of oxygen and carbon dioxide from the gas sample, and for blood reduction.

*Neutral ferricyanide reagent* 20 gm of potassium ferricyanide and 8 gm of saponin<sup>1</sup> are dissolved in water to make 100 cc of solution. If kept in a glass-stoppered bottle, the reagent may be used for 2 weeks.

*Air-free 1 N sodium hydroxide solution* Approximately 40 gm of NaOH are dissolved in water to make 1 liter of solution. This is extracted air-free and kept over mercury (4) in a Sendroy (6) vessel.

*Glycerol-salt solution* 1 volume of glycerol is mixed with 3 volumes of saturated sodium chloride solution (14).

*Caprylic alcohol* This is used to prevent foaming.

*Fresh beef blood* is reduced as described below.<sup>2</sup>

### Procedure

In the following, the description is largely confined to an outline of the general procedure, details being given or stressed only when they constitute modifications of, or departures from, the original technique (5), to which the reader desirous of using the method should refer for more complete and necessary information.

*Admission and Measurement of Gas Sample*—Approximately 35 cc of are admitted into the cleaned and empty chamber of the Van Slyke-Neill apparatus, and measured by the pressure readings  $p_0$  and  $p_1$ , taken at the 50 cc volume, before and after the admission of the gas sample ((15) pp 512-518).

*Deoxygenation of Gas Sample*—5 cc of the alkaline hyposulfite solution are placed in the cup, 3 cc are allowed into the chamber, and the stopcock is sealed with mercury. The gas sample is slowly shaken with this solution at slight negative pressure ((15) p 521) for 3 minutes. The residual gas<sup>3</sup> is transferred from the apparatus, through a mercury seal,

<sup>1</sup> After this work was completed, we learned that owing to the war saponin may be unobtainable. Preliminary experiments indicate that, under the conditions of analysis to be described, it may be omitted from the ferricyanide reagent, without affecting the results.

<sup>2</sup> Fresh material may not always be available. However, we have usually found it possible to preserve the same lot of beef blood with its capacity for CO absorption unimpaired for 1 week, by storage in the refrigerator in portions completely filling several 50 cc stoppered flasks. A previously unopened flask was used for each day's analyses. The blood used in this work was supplied by The Armour Laboratories, through the courtesy of Dr J H Glynn.

<sup>3</sup> The hyposulfite used here absorbs all of the  $\text{CO}_2$  in the sample, but does not quite completely deoxygenate the latter, as is shown in another section. The previously

into a modified (stop-cock) Hempel pipette (Van Slyke and Hiller (10)) or a Sendroy ((6) Fig 1, Model 3) gas-sampling vessel, containing glycerol-salt solution<sup>4</sup> The technique used in transferring the gas is that described by Sendroy and Liu ((8) p 136) The hyposulfite solution is allowed to ascend into the arm of the Hempel pipette until it *just* enters the bore of the stop-cock, which is then turned so that the capillary may be flushed with mercury from the cup above The hyposulfite is then ejected from the chamber, so that only the film adhering to the walls remains

*Reduction of Blood*—A 5 cc ( $\pm 0.1$  cc) portion of beef blood (at room temperature) is admitted into the unwashed chamber, followed by 5 cc of water and 2 drops of caprylic alcohol The blood is then reduced and deaerated by two extractions *in vacuo* of 3 and 2 minutes each, as previously described ((5) pp 601-602), except that 2 drops instead of 1 of hyposulfite solution are used for the second shaking and extraction The liberated gas is ejected after each extraction as usual, without loss of fluid

*Absorption of Carbon Monoxide*—The deoxygenated gas stored in the container vessel is returned to the chamber over the reduced blood, and the CO is absorbed, with exclusion of light,<sup>5</sup> by slow shaking (approximately 200 RPM) for 30 minutes at slightly negative pressure The upper portion of the chamber is then temporarily uncovered, while the unabsorbed gas is ejected, and 4 drops of caprylic alcohol are added ((5) p 602) With the light shield replaced, the dissolved gases (including nitrogen and a mere trace of CO) in the diluted blood mixture are then -extracted by rapid shaking *in vacuo*, for 5 minutes The shield is removed, and the extracted gas is ejected

*Liberation and Measurement of CO Absorbed by Blood*—From a stop-cock pipette, through a mercury seal, 10 cc of the ferricyanide solution is admitted into the chamber, directly over the diluted blood The evacuated chamber is shaken for  $7\frac{1}{2}$  minutes<sup>6</sup> The CO<sub>2</sub> evolved is then

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suggested additional measurement of O<sub>2</sub> (+ CO<sub>2</sub>) in the gas sample is therefore inapplicable ((5), foot-notes 1 to 3) to the method of the present paper

<sup>4</sup> Transfer of the gas to containers of this type eliminates the possible inconvenience of the attached gas-sampling tube used in the original technique ((5) p 600) When the Sendroy vessel is used, the vertical capillary (a<sub>2</sub>) of the stop-cock is surmounted by a small tube or other suitable device, attached by means of rubber tubing (o) and corresponding to the cup of the Van Slyke-Hiller-Hempel pipette Accumulations of mercury at the junction of the container bulb and the end of the separatory funnel tube are drawn up into the latter by gentle suction applied to the neck of the funnel The vessel is then tilted and the mercury poured off through the separatory funnel

<sup>5</sup> Tin-foil or any opaque paper or cardboard may be used to cover the jacket of the apparatus

<sup>6</sup> At temperatures lower than 20°, CO is liberated from hemoglobin more slowly (2) A 10 minute extraction period is then required

absorbed with 1 cc of air-free 1 N NaOH, and the CO is measured at 0.5 cc volume ((10) p 812, (13) pp 545-546). The readings  $p_2$  and  $p_3$  correspond, respectively, to the pressures before and after the ejection of the residual gas (CO) from the apparatus.<sup>7</sup>

*Blank Analysis, Determination of "c" Correction*—This analysis ("whole" blank) is carried out exactly as described above, except that approximately 35 cc of laboratory air (CO-free) are used in place of the unknown CO-containing sample. The readings  $p'_2$  and  $p'_3$ , respectively, are taken before and after the final ejection of residual gas from the apparatus.

However, as will be shown, the above procedure may usually be shortened and the resultant "simple" blank used with a correction, to effect considerable economy of time and labor without loss of accuracy. Thus, after deoxygenation of the blank air sample, the remainder of the air (nitrogen), instead of being transferred to a container vessel, is discarded together with the 3 cc of used hyposulfite absorbent. 5 cc of blood, 5 cc of water, and 6 drops of caprylic alcohol are then made air-free by successive 3 and 2 minute extractions, with added hyposulfite, as above. After the second ejection of extracted air, *the equilibration of the reduced blood with deoxygenated gas (as described above) is here omitted*. Consequently, there immediately follows another extraction *in vacuo*, for 5 " " " " with the light shield in place. Any gas thus liberated is ejected, the analysis is continued and finished as above ("Liberation and measurement of CO absorbed by blood"). The final readings in this procedure are designated by the symbols  $p''_2$  and  $p''_3$ .

In the analysis of some industrial gases, an additional correction for dissolved gases other than air may sometimes be required. The procedure in such cases is outlined at the end of this paper.

### Calculation

The pressure of the sample at 50 cc volume is calculated as

$$(1) \quad P_s = p_1 - p_0$$

The CO pressure at 0.5 cc volume is calculated as

$$(2) \quad P_{CO} = p_2 - p_3 - c$$

The "whole" blank correction term,  $c$ , is obtained from readings in blank analyses carried out as described above. Thus, depending on

<sup>7</sup> For these readings, the bottom of the meniscus may not be clearly observable through the blood-ferricyanide mixture. However, partial visibility through the layer of NaOH added, together with experience in reading a water meniscus, should enable the operator to estimate and adjust the position of the bottom of the meniscus accurately.

whether the "whole" blank or the "simple" blank is determined analytically, the correction is calculated, respectively, as either

$$(3) \quad c = p' - p'_2 \text{ or}$$

$$(4) \quad c = p''_2 - p''_1 + 1.0$$

Since composition of the gas is calculated as

$$(5) \quad \% \text{ CO} = \frac{100 \times \text{cc CO in sample}}{\text{cc volume of sample}}$$

pressures are converted to volumes in the equation

$$(6) \quad \% \text{ CO} = \frac{f_1 P_{\text{CO}}}{f_2 P_s}$$

in which  $f_1$  is the factor by which  $P_{\text{CO}}$ , measured at the 0.5 cc volume and the observed temperature, is multiplied to give 100 times the volume, in cc at 0°, 760 mm, of carbon monoxide present in the gas sample analyzed, and  $f_2$  is the factor by which  $P_s$ , measured at the 50 cc volume and at the observed temperature, is multiplied to give the volume in cc at 0°, 760 mm, of gas sample used. When  $P_{\text{CO}}$  and  $P_s$  are measured at the same temperature, the composite factor  $f_3 (= f_1/f_2)$  is used.

The derivation of the above factors has been given by Sendroy (5). Values for  $f_1$  have been recalculated for this paper in accordance with the change in conditions of analysis prescribed ( $S$  now = 11 cc instead of 18 cc, and the "correction factor" is now 1.020 instead of 1.067), and are given in Table I, together with those for  $f_2$  and  $f_3$ .

Except when it is desired to know the volume ( $\times 100$ ) of CO in the sample, or the actual volume of the sample, the use of factors  $f_1$  and  $f_2$  is unnecessary, and the calculations are simplified, with loss of accuracy not greater than 0.1 per cent, as follows. For readings within the ranges of temperature 10–16°, 17–28°, and 29–34°,  $P_{\text{CO}}/P_s$  is simply multiplied by the factor  $f_3$ , 1.028, 1.027, or 1.026, respectively. For each 1° of temperature difference between the readings for  $P_{\text{CO}}$  and  $P_s$ , the foregoing  $f_3$  factors are first corrected by 0.3 per cent, plus or minus, depending on whether  $P_{\text{CO}}$  is measured at a temperature lower or higher, respectively, than that of  $P_s$ .

*Example*—Readings were  $p_0 = 7.1$  mm,  $p_1 = 493.8$  mm, at 23.8°, at 50 cc volume, and  $p_2 = 194.0$  mm,  $p_3 = 66.0$  mm, at 24.6°, at 0.5 cc ( $a = 0.5025$  cc) volume. The "simple" blank readings were  $p'' = 83.2$  mm,  $p''_2 = 66.0$  mm. The calculation (with correction for  $a$ ) was as follows:

$$\begin{aligned} \% \text{ CO} &= \frac{(128 - (17.2 + 1.0))}{486.7} \times (1.027 - (0.003 \times 0.8)) \times \frac{0.5025}{0.5000} \\ &= \frac{109.8}{486.7} \times 1.025 \times 1.005 = 0.232 \end{aligned}$$

TABLE I

*Factors for Calculation of Results from Pressure Measurements in Analysis of CO in Gas Mixtures*

Temperature	Factor $f_1$	Factor $f$	Factor $f_2$
C			
10	0 0652	0 0634	1 028
11	49	31	
12	47	29	
13	44	27	
14	42	24	
15	39	22	
16	37	20	
17	35	18	1 027
18	32	15	
19	30	13	
20	27	11	
21	25	09	
22	23	07	
23	21	05	
24	19	02	1 026
25	16	00	
26	14	0 0598	
27	12	96	
28	10	94	
29	08	92	
30	05	90	
31	03	88	1 026
32	01	86	
33	0 0599	84	
34	97	82	

## EXPERIMENTAL

In principle, the types of experiments performed in establishing the accuracy of the method and the range of precision were the same outlined previously (5) and consisted of the preparation and analysis of air samples containing added CO in amounts either known or measured by independent analyses. In certain details, however, the procedures were different from the above, as will be described in the following.

*Analyses of Air Containing Known Amounts of Added Pure CO*—In this group of experiments, the results of which are recorded in Table II, the gas samples were made by the accurate dilution of pure CO gas in CO-free air, to give mixtures containing from 0.05 to 0.8 per cent CO.

((5) p 608) The mixtures were prepared as described by Van Slyke and Sendroy (15), and Van Slyke, Sendroy, and Liu (16) The purity of the several lots of CO used, made from formic and sulfuric acids, was con-

TABLE II  
Results of 53 Analyses of Air Containing Known Amounts of Added Pure CO

Analysis group No	Air sample No	Ox blood lot No	No of analyses	Per cent CO in air	Ratio of per cent CO found by analysis to that present	Average of ratio for group	Deviation from average ratio of 0.980*
I	1	1, 2	6	0.0571	0.984	0.980	$\pm 0.018$
	2	3	2	0.0523	0.969		
II	3	4, 5, 6	5	0.0905	0.999	0.990	$\pm 0.016$
	4	6, 7	6	0.0875	0.989		
	5	8	3	0.0955	0.983		
	6	9	2	0.1036	0.982		
III	7	10	2	0.217	1.001	0.979	$\pm 0.011$
	8	11	2	0.207	0.962		
	9	12, 13	7	0.187	0.974		
	10	14	1	0.238	0.985		
	11	15	1	0.277	0.991		
	12	16	1	0.218	0.972		
	13	16	1	0.238	1.000		
	14	17	1	0.269	0.982		
	15	17	1	0.169	0.975		
	16	18	1	0.198	0.961		
	17	19	1	0.214	0.985		
IV	18	20	1	0.585	0.975	0.978	$\pm 0.005$
	19	20	1	0.627	0.982		
	20	20	1	0.591	0.983		
	21	20	1	0.694	0.983		
	22	20	1	0.718	0.979		
	23	21	1	0.716	0.973		
	24	21	1	0.822	0.979		
	25	21	1	0.813	0.960		
	26	21	1	0.632	0.983		
	27	21	1	0.790	0.985		
Average						0.982	$\pm 0.013$

\* The value 0.980 represents the average of the ratio for CO found to CO present, for all the results of Tables II and III

trolled in analyses by absorption with Winkler's cuprous chloride solution. Five such analyses gave values of 99.1, 100.4, 99.5, 100.0, and 99.2 per cent CO. Since the indicated average impurity of 0.4 per cent was well within



the limit of error of the analyses, no correction was made for it in the preparation of known CO gas mixtures. The air used for dilution was controlled by a method as yet unpublished, developed in this laboratory, and sensitive to 0.001 per cent of CO in air. The results of repeated tests of the room air used in the preparation of known CO mixtures were consistently negative.

*Analyses of Air Containing Measured Amounts of CO Extracted from Blood*—In this group of experiments, the results of which are recorded in Table III, CO was extracted from blood in the Van Slyke-Neill apparatus,

TABLE III

*Results of Fifteen Analyses of Air Containing Measured Amounts of CO Extracted from Blood*

Air sample No	Ox blood lot No.	Per cent CO in air	Ratio of per cent CO found by analysis to that present	Deviation from average ratio of 0.980*
1	22	0 232	0 981	+0 001
			0 981	+0 001
			0 982	+0 002
			0 982	+0 002
			0 976	-0 005
			0 974	-0 006
			0 973	-0 007
			0 989	+0 009
2	23	0 216	0 955	-0 025
			0 972	-0 008
			0 955	-0 025
			0 968	-0 012
			0 976	-0 004
			0 964	-0 016
			0 984	+0 004
Average			0 974	±0 008

\* The value 0.980 represents the average of the ratio for CO found to CO present for all the results of Tables II and III.

measured, and then used directly in the preparation of 0.2 per cent mixtures in air ((8) pp 144-145, (5) pp 608-609). The procedure followed, with slight modifications, was patterned after the blood CO capacity method of Van Slyke and Hiller (10). Samples of 3 cc of blood, with 6.25 cc of water added, were equilibrated in the extraction chamber with 2.5 cc of CO, by shaking for 5 minutes with the mercury level at the 50 cc mark. The extracted gases and excess CO were ejected. The CO retained by the blood was then liberated by the addition of 0.75 cc of acidified potassium ferricyanide (20 per cent  $K_3Fe(CN)_6$ ) and 2 per cent

saponin in 2 per cent lactic acid) and extraction *in vacuo* with shaking for 5 minutes

For the measurement of CO liberated from the saturated blood, the procedure was continued as follows. After the addition of 1 cc of air-free 1 N NaOH, 1 cc of air-free alkaline hyposulfite (in 1 N KOH with catalyst (Van Slyke (9) p 124)) was added and a reading  $p_1$  taken at 20 cc volume, in the usual way (13, 9)<sup>8</sup>. The gas was ejected, and the reading  $p_2$  was recorded. The "c" correction for the small amount of nitrogen liberated from the ferricyanide reagent, and therefore included in the measurement of  $p_1$ , was found by a repetition of the above procedure with an equal volume of water in place of blood, and the omission of CO, for the first 5 minutes shaking and extraction. Thus, this method provided data for the calculation

$$P_{CO} = p_1 - p_2 - c$$

From this there was obtained, by the use of a factor corresponding to  $f_2$  for 20 cc volume, the value of  $V_{CO}$ , the volume of free CO at 0°, 760 mm, extracted from the 3 cc blood sample and available for dilution with air after its measurement at the 20 cc volume

For the preparation of gas samples containing the same amounts of CO thus extracted and measured, the above procedure was repeated with the following changes. At the end of the 5 minute period of extraction with ferricyanide, with the mercury level still at the 50 cc mark, a calibrated gas-sampling tube was attached to the capillary side arm (16). Alkali and hyposulfite were added to the contents of the extraction chamber, as above, and the reading  $p_1$  taken at 20 cc volume. The mercury level

<sup>8</sup> The addition of alkaline hyposulfite after the introduction of the 1 N NaOH at this point was a departure from the technique of Van Slyke and Hiller (10), necessitated by the fact that we did not find it possible to obtain complete saturation of the blood hemoglobin with CO under the conditions outlined above and prescribed by them. As a result, since some oxygen, not displaced by CO, was always released by the ferricyanide, the subsequent measurement indicated  $O_2 + CO$ , unless hyposulfite was used to absorb the former. The small CO reabsorption effect of the hyposulfite ((13) p 563), on the other hand, was of no consequence in the accurate measurement, after reabsorption, of the amount of CO diluted in the mixture with air. These observations do not bring into question the validity of the method of Van Slyke and Hiller (10) as a true measure of active hemoglobin, the measured capacity for ( $O_2 + CO$ ) being in all cases the same as that for CO capacity. In our investigation of this point, we have found that if the time of their equilibration with CO is extended from 1 minute to 7 minutes, all of the gas bound by the hemoglobin is CO. A more recent modification of their CO capacity method (Van Slyke and Hiller (12)) likewise insures complete saturation of the blood with CO over a 1½ minute equilibration period.

was then lowered, and air was admitted to the chamber for the dilution of the CO and transfer of the mixture to the gas-sampling tube

The reading  $p_1$  thus observed provided a check of the value obtained in the analysis described above. Although no  $p_2$  reading was here available, it could be assumed to be the same as that observed in such previous measurement, if no significant temperature change had taken place in the interim. The final volume of the gas mixture in the tonometer was adjusted and calculated in the usual way (15, 16)

*Results Evaluation of Correction Factor 1.020*—The average of all the results of Tables II and III for the analysis of CO in air mixtures containing 0.05 to 0.8 per cent of that gas, by the techniques described, is  $98.0 \pm 1.2$  ( $\sigma$ , or  $s_D$ ,  $\pm 1.5$ ) per cent of the true concentrations (known, or obtained by independent measurement). By statistical analysis each of the average values of the ratio, found to present, for the groups of Table II and for Table III is significantly different from the perfect value of 1.000. On the other hand, the differences among the values themselves are not significant, either with variation in CO concentration (97.8 to 99.0 per cent for the group averages of Table I), or with difference in the type experiment employed (averages of 97.9 and 97.4 per cent, respectively, the comparable 0.2 per cent CO groups Group III, Table II, and Table III). The corresponding empirical correction factor 1.020 ( $1/0.980$ ) has therefore been incorporated in the factor  $f_1$  (Table I) for the calculation of the CO concentration or content, of gas mixtures analyzed by the present method.

*Relationship of "Simple" Blank to "Whole" Blank Corrections*—In order that  $P_{CO}$  may truly represent the CO initially in the gas sample (or rather, 98.0 per cent of it) the readings  $p_2$  and  $p_3$  actually obtained must be corrected for the presence of other gases extracted in the final  $7\frac{1}{2}$  minutes shaking with ferricyanide, namely, (a) the slight amount of bound CO present in normal blood, (b) dissolved air or nitrogen in the added ferricyanide reagent, and (c) that portion of the nitrogen absorbed by the diluted blood during the 30 minute equilibration with deoxygenated air, which is not liberated in the following 5 minutes extraction. The "whole" blank provides a complete correction for all of these components of error (Equations 2 and 3)

Since the CO content of normal blood is variable and its determination requires the use of ferricyanide, the corrections for components (a) and (b) must be determined daily for samples of the same blood and ferricyanide used for analysis. With the elimination from the "whole" blank determination, of the 30 minute equilibration of blood with deoxygenated air, there is provided by the resultant "simple" blank a control of components (a) and (b). Component (c), as might be expected, is so constant

(and small) that, once determined, the same correction for it may usually be used for all analyses. The average value for this correction, in a series of fourteen comparisons of "whole" and "simple" blank analyses, was found to be 1.0 mm, measured at 0.5 cc volume (Table IV). Thus, this value, when added to the determined "simple" blank result, gives the "whole" blank correction (Equation 4). This simplified procedure is recommended for the analysis of all air samples except those containing CO in the region of 0.05 per cent. Although the average deviation from the above value of 1.0 mm is only  $\pm 0.3$  mm, a possible *maximum* deviation of  $\pm 1.0$  mm would involve an error of  $\pm$  per cent at this concentra-

TABLE IV  
Comparison of "Whole" and "Simple" Blank Analyses

Good sample No	Pressure in mm at 0.5 cc volume for			
	"Whole" blank W	"Simple" blank S	Difference between blanks W - S	Deviation from average difference of -1.0
1	22.1	20.7	-1.4	-0.4
	22.2	21.1	-1.1	-0.1
	22.8	21.3	-1.5	-0.5
	23.1	22.2	-0.9	-0.1
	22.0	21.5	-0.5	-0.5
2	15.0	13.4	-1.6	-0.6
	18.8	17.2	-1.6	-0.6
3	20.7	20.0	-0.7	+0.3
	21.1	20.1	-1.0	0.0
4	18.1	17.4	-0.7	-0.3
	18.3	17.1	-1.2	-0.2
5	18.7	18.7	0.0	+1.0
6	21.1	20.1	-1.0	0.0
7	20.8	19.8	-1.0	0.0
Average			-1.0	$\pm 0.3$

tion of CO. Hence, for such samples, the "whole" blank correction should be determined directly.<sup>2</sup>

*Factors Affecting Results*—For the method described in this paper, the extent to which the CO found by analysis is a true measure of that present in the air sample is determined by several factors of error which are not

<sup>2</sup> An obvious further simplification of the "simple" blank analysis would be the omission of the initial step, namely, the deoxygenation of the air sample which is immediately discarded. For some unknown reason, however, we have not found it possible to establish a sufficiently constant relationship between the results of such blanks, and of "whole" blanks.

controlled by the blank analyses. The magnitude of these errors depends on the extent of deviation from the following theoretical requirements, (1) the complete elimination from the blood mixture of any oxygen, either bound (as oxyhemoglobin) or dissolved, which might be evolved upon the addition of ferricyanide and subsequently be measured and accounted for as carbon monoxide, and (2) the complete absorption by the blood of the CO in the air sample, and its subsequent quantitative liberation and measurement.

1 The hyposulfite mixed with the blood reduces the oxyhemoglobin and removes the oxygen as such, by chemical reaction. Preliminary deoxygenation of the air sample avoids the presence of a large amount of extra oxygen possibly interfering with the complete reduction of the hemoglobin. Because the alkaline hyposulfite used in this method was of different composition from that previously used,<sup>10</sup> its efficiency in this respect was tested by its use in the analysis of air for oxygen (15). The results showed that 0.6 per cent  $O_2$  was left unabsorbed in the gas sample. Apparently, however, when "deoxygenated" gas containing this amount of unabsorbed  $O_2$  is equilibrated with blood, the hyposulfite previously added to the blood removes the oxygen and prevents the formation of oxyhemoglobin. That this is so is proved by the fact that the difference between the analysis of the "whole" blank, in which the blood is equilibrated with a residual air sample, and the "simple" blank, in which it is not, amounts to only 1 mm, a difference which, by calculation, is wholly attributable to the unextracted dissolved nitrogen. It is reasonable to conclude, therefore, that no oxygen is liberated from the blood and included in the readings for  $P_{CO}$  in the present method.

2 The ratio, 0.980, of CO found by analysis to CO present in air samples is 4.3 per cent higher than that found by the method as originally developed by Sendroy (5), who suggested that differences in quantitative technique or control might to some extent be responsible for the variation in results obtained by different workers using this principle in quantitative work. Although the method of CO measurement employed in the present modification is more convenient and easier to carry out, it is slightly less accurate (Roughton (2)) than that (Sendroy and Liu (8)) used in the determination of the ratio 0.937 (5). However, the possible difference in accuracy of the two methods of measurement would fail to account for the increase in recovery of from 93.7 to 98.0 per cent. The change, therefore, must represent a real increase in the proportion of the total CO of

<sup>10</sup> In order to retard the rapid deterioration of grease in the stop-cock below the cup, and the consequent leakage of air into the extraction chamber, sodium hydroxide was used in place of potassium hydroxide, and the concentration of hyposulfite reduced to one-half that used by Van Slyke and Sendroy (15) for oxygen analysis.

the gas sample, absorbed by the blood. That this is indeed the case is shown by the results of the experiments in Table V. Either value, 0.940 or 0.980 respectively, could be obtained, depending solely on whether the blood used for equilibration with the gas sample was undiluted, as in Sendroy's procedure, or diluted as in the present modification. Analyses of diluted samples by the technique of Sendroy (5) confirmed this effect. Apparently, the dilution increases either the affinity of hemoglobin for CO under these conditions, or the rate of absorption of the gas. Thus, no other variable, such as the percentage of CO in the gas sample, or the volume or types of reagents (Table V), or the method of measurement of the CO, is a factor in this respect.

TABLE V

*Effect of Dilution with Water on Absorption by Blood of CO from Air*

Air sample No	Per cent CO in air	Volume				Ratio of per cent CO found by analysis to that present
		When CO absorbed		When CO extracted		
		Blood	Water	Fern cyanide reagent	Total fluid (S)	
5	0.0955	5	0	1	6	0.930
6	0.1036	5	5	1	11	0.983
		5	0	1	6	0.930
		5	5	1	11	0.982
A	0.3	5	0	13*	18	0.941
B	0.8	5	0	13*	18	0.940
		5	5	8*	18	0.984
All in Sendroy's (5) experiments	0.05-0.3	5	0	13*	18	0.937
All in present paper	0.05-0.8	5	5	1	11	0.980

\* Reagent acidified with lactic acid

*Application of Method to Analysis of Industrial Gases*—In the procedure developed, CO is analyzed in gas samples composed mainly of air. This condition holds in the analysis of most industrial gases because their high CO content (illuminating gas 2 to 30 per cent, automobile exhaust gas 5 to 7 per cent) requires dilution with air as a preparation for analysis by this method, the upper limit of the range of which is set at 0.8 per cent CO. However, the possible effect of other gases present, such as methane, ethane, and other saturated hydrocarbons, and also ethylene, acetylene, and other unsaturated hydrocarbons, must be considered. Although these gases do not combine with hemoglobin, they may, because of their higher solubilities, be absorbed in correspondingly large amounts, even when diluted with air. Portions not completely liberated

in the 5 minute extraction period prior to the addition of ferricyanide, when liberated in the final extraction, would be indistinguishable from CO. The blank determinations with *atmospheric* air do not correct for such errors.

To test this point, we have carried out control analyses with several air-diluted samples of illuminating (a mixture of natural and artificial) gas and automobile exhaust gas. The procedure for the analysis of the unknown gas mixture and the determination of the "whole" blank with air were carried out exactly as described above, except that in each case 10 cc of water were used instead of the diluted blood mixture. The dif-

TABLE VI

*Corrections Found for Dissolved Gases Other than Air in Analysis of Industrial Gases for CO*

Gas sample No	Mixture	No. of extractions before addition of $\text{K}_3\text{Fe}(\text{CN})_6$	Correction to be subtracted from $P_{\text{CO}}$ values
			mm
1	Illuminating gas* 1 part, air 1 part	1	5.1
2		2	1.5
3		2	1.9
4	Illuminating gas* 1 part, air 9 parts	1	1.1
5		1	1.2
6		2	0.7
7	Automobile exhaust gas† 1 part, air 9 parts	1	0.3
8		1	0.0
9		1	0.4

\* Containing in volumes per cent, CO 2.5, carbon dioxide 1.0, unsaturated hydrocarbons 2.0, oxygen 0.5, hydrogen 24.5, methane 55.0, other saturated hydrocarbons 7.0, and nitrogen 7.5. Approximate values based on analyses supplied by courtesy of the Peoples Gas Light and Coke Company, Chicago.

† Containing approximately 6 per cent CO.

ference in pressures was taken as the *correction for dissolved gases other than nitrogen*, unextracted in the 5 minute extraction period preceding the extraction with ferricyanide. Table VI indicates that this correction will be small for most analyses of industrial gases, and that in the unusual circumstances in which it may be greater than 1 or 2 mm it may be reduced by a second extraction of 3 minutes and ejection of gas, prior to the addition of ferricyanide. It is therefore recommended, when mixtures such as these are diluted no more than 10 per cent with air, or when there is reason to suspect the presence of higher concentrations of a highly soluble gas such as acetylene, that the operator determine the correction for the particular type of gas mixture analyzed, as described. Once

determined, it may thereafter be applied to all analyses of that gas as a subtraction from the results ( $P_{\infty}$ ) obtained as usual. It should be noted that when a second (3 minute) extraction period is required to lower this correction, the added step in the procedure is uniformly carried out for the actual gas analysis, and the "whole" blank determination, also.

From the foregoing, it is apparent that the procedure may be applied to the analysis of industrial gases initially containing even as little as 0.5 per cent of CO, if they are first diluted to 10 times volume with air. Such gases will probably be rarely encountered.

#### SUMMARY

A modification of Sendroy's method is described, whereby small amounts of carbon monoxide in air and other gas mixtures, in concentrations of from 0.05 to 0.8 per cent, are analyzed in the Van Slyke apparatus. By a change in reagents, procedure, and technique of measurement, there have been effected an economy of time and labor and an increase in accuracy and precision of the results. The method is applicable to the analysis of industrial gases.

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# THE DEPOSITION OF CALCIUM, PHOSPHORUS, AND CARBON DIOXIDE IN CALCIFYING DENTAL ENAMEL

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The properties of dental enamel suggest that it may have several advantages over bone for chemical studies of the process of calcification. First, it is histologically homogeneous, uniform and comparable samples can be obtained for analysis. Secondly, it calcifies *en masse* by infiltration of inorganic material into the substance of a preformed organic matrix (1). Deposition of the mineral phase begins at the cusp tips of this organic pattern and spreads gradually over the whole crown of the tooth, producing a calcification gradient. Anatomically, this gradient is large enough to provide a series of samples in various degrees of calcification, each of which can be analyzed by microchemical methods. Thirdly, calcifying enamel undergoes neither simultaneous resorption nor growth of the matrix, processes which complicate the interpretation of bone analyses. For these reasons we believe that the chemical changes to be found in developing enamel more accurately describe the fundamental process of calcification than those found in bone. In this study we have determined the Ca, P, and CO<sub>2</sub> contents of pig enamel at various levels of calcification and followed the quantitative changes in each during the course of development.

In a previous study (2) it was found that the absolute amount (mg per c mm) of inorganic substance in pig enamel was proportional to the specific gravity of the fresh, undried tissue. Therefore, specific gravity could be used as a measure of the degree of calcification and plotted against the results of the microchemical analyses expressed in mg per c mm. Thus, the absolute amounts of ash, organic material, water, Ca, P, and CO<sub>2</sub> per unit volume of enamel can be correlated with the degree of calcification in each sample analyzed. Used with any calcified tissue, this method eliminates the errors inherent in calculating composition as percentage of ash or dry weight. When applied to enamel it minimizes the effects of any secondary changes in calcification brought about by age, growth and resorption, the nutritional state, and individual differences between animals. The details of the analytical method have been reported (2).

## EXPERIMENTAL

*Preparation of Material*—Unerrupted molars of pigs were extracted at the abattoir and taken immediately to the laboratory for analysis. The enamel organ was removed, the tooth washed with water, and samples of enamel (5 to 20 mg) at various stages of calcification were chipped off with a scalpel (2). The samples were placed in vials with a few drops of

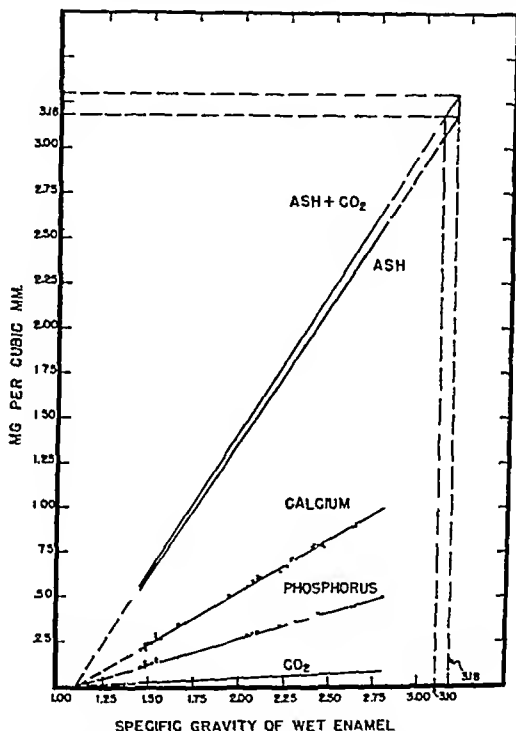


FIG 1 Ca and P plotted against the specific gravity of fresh, undried enamel. The  $\text{CO}_2$  and ash curves have been transcribed from Fig 2 and reference (2), respectively, for comparison.

distilled water and left in the refrigerator to await analysis. Each sample was then blotted with a moist chamouis to remove visible water and weighed on the micro balance. The specific gravity was determined by flotation in a mixture of bromoform with chloroform or petroleum ether (2).

*Chemical Methods Carbonate*—Samples for the determination of carbonate were given this preparatory treatment and placed in Warburg vessels for analysis. The carbonate was determined as  $\text{CO}_2$  by a modifi-

cation of the direct Warburg method (3) The technique was standardized as follows Micro samples of calcite (about 0.7 mg) were weighed on a micro balance to  $\pm 5 \gamma$  and carefully transferred to the side bulbs of standard Warburg vessels 2 cc of 2 N HCl were pipetted into the main chamber Following an equilibration period of 15 minutes, the calcite was dumped into the acid and the manometers read after 1 hour The readings were multiplied by vessel constants with no correction for  $\alpha_{\text{CO}_2}$  The  $\alpha_{\text{CO}_2}$  for 2 N HCl could not be found in the literature In a series of twenty-five determinations on calcite the average yield of  $\text{CO}_2$  was 92.6 per cent of the theoretical, with an average deviation of  $\pm 1.2$  per cent Therefore, the vessel constants were increased by 7.4 per cent to compensate for the effect of  $\alpha_{\text{CO}_2}$  for 2 N HCl All determinations were made at  $25^\circ \pm 0.1^\circ$  One manometer was used as a thermobarometer The vessels were shaken at the rate of 75 complete oscillations per minute After the standardization, forty-eight enamel samples were analyzed by the same procedure

*Calcium and Phosphorus*—Thirty-one samples were selected at random, placed in volumetric flasks of suitable size, and dissolved in 1 cc of 5 per cent HCl When decalcification was complete, the solution was made up to volume with water and calcium (4) and inorganic phosphorus (5) determinations made on duplicate aliquots

*Total Inorganic Material*—For convenience the ash plus  $\text{CO}_2$  is called "total inorganic material" The ash curve in Fig. 1 was taken from previous ashing data (2), the  $\text{CO}_2$  curve was drawn from the  $\text{CO}_2$  determinations described above The sum of these two gave the top curve in Fig. 1 from which the formula

$$\text{Total inorganic material} = (\text{sp. gr.} - 1.10) \times 1.58$$

was derived graphically and used for calculating the inorganic content of individual samples 1.58 is the slope and 1.10 the specific gravity intercept when the curve is extrapolated to zero ash

*Calculations*—The volume of each sample was derived from the wet weight and specific gravity All primary data for Ca, P, and  $\text{CO}_2$  values were calculated in mg per c mm for reasons previously explained (2)

### Results

The average composition of the inorganic material in pig enamel is given in Table I The data for Ca, P, and  $\text{CO}_2$  are shown in Figs. 1 and 2 Although the specific gravity of the samples varied from 1.45 to 2.86, the Ca, P, and  $\text{CO}_2$  contents of the inorganic material remained quite constant

From this and previous work (2) it was found that the specific gravity

of 125 samples of the calcifying enamel ranged from 1.45 to 2.86. Fifteen of the samples were below 1.50, but none was found below 1.45. This is negative evidence, at least, that the organic matrix is deposited by the

TABLE I  
*Average Composition of Inorganic Material\* in Pig Enamel*

	No. of analyses	Per cent of inorganic material	Atomic ratios
Ca	31	38.0 $\pm$ 1.1	13.6
P	31	19.3 $\pm$ 0.7	8.9
CO <sub>2</sub>	48	3.1 $\pm$ 0.22	1.0

\* Ash + CO<sub>2</sub>

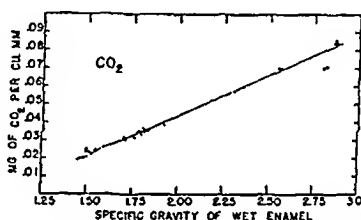


FIG. 2 The amount of CO<sub>2</sub> per c mm of fresh, undried enamel plotted against specific gravity

TABLE II

*Composition of Enamel at Extremes of Calcification Derived Graphically from Fig. 1*

	Lowest calcification sp gr = 1.45		Highest calcification sp gr = 2.80	
	mg per c mm	per cent of wet weight	mg per c mm	per cent of wet weight
Inorganic*	0.56	38.4	2.68	95.7
Ash (2)	0.54	37.0	2.60	92.8
Ca	0.22	15.2	1.00	35.7
P	0.11	7.6	0.50	17.9
CO <sub>2</sub>	0.02†	1.4	0.08	2.9

\* Ash + CO<sub>2</sub>

† 0.018 on original chart, scale 0.1 inch = 0.002

ameloblasts, partially calcified with a composition corresponding to a specific gravity of 1.45. As calcification of the matrix proceeds to a specific gravity of 2.80 or more, the ash, Ca, P, and CO<sub>2</sub> all increase linearly (Fig. 1). The composition at the two extremes is shown in Table II. If the curves for all the constituents are extrapolated to zero ash, they

intersect the abscissa approximately at the same point, indicating that the various ratios are practically constant throughout the range of calcification shown. The Ca P ratio, for example, is 2.00 when derived graphically, calculated from thirty-one analyses it is 1.97 with an average deviation of  $\pm 0.04$ . While the other graphically derived ratios obtainable from Table II are not so constant between the lowest and highest degrees of calcification, yet neither Ca nor  $\text{CO}_2$  increases relative to P or ash.

If the ash curve in Fig. 1 is extrapolated in the other direction, a point is reached where the ash and specific gravity values become equal. This point, which is at a specific gravity of 3.18, represents an enamel whose composition is 100 per cent ash. If the "ash +  $\text{CO}_2$ " curve is then extrapolated to 3.18 and this point projected to the specific gravity axis, it is found that the total inorganic material (ash +  $\text{CO}_2$ ) has a specific gravity of 3.10. This value compares favorably with the value 3.055 found in human enamel by x-ray diffraction methods (6). Considering the small amount of other inorganic elements present, this is a reasonable value for the specific gravity of the mineral present in pig enamel.

#### DISCUSSION

In terms of tooth development, the range of calcification studied here covers all stages from the softest to the hardest enamel found in twenty or more teeth of pigs at or before eruption. The increase in ash is 5-fold, yet the ash Ca P  $\text{CO}_2$  ratios are all essentially constant. Because there is no mechanism analogous to that in bone for withdrawal of the inorganic salts from enamel, one may assume that these constituents are continuously deposited in the same proportion found here. If this is true, the carbonate is not deposited independently of the phosphate, it must be in some combination with the phosphate or its deposition is controlled by the same factors. Claims that the proportion of these three elements in enamel changes with time are based on analyses of whole teeth (7) (in which the dentin-enamel ratio may also vary), on comparisons of fetal with adult teeth (8), or isolated analyses by different investigators (8).

Calcification studies based on bone analyses seem to show that Ca and  $\text{CO}_2$  increase relative to P as the bone grows older. The chemical determinations have been made, however, on whole bones or powdered samples of whole bones at different ages (9-11, 12). Since a calcifying bone is a constantly changing mixture of cartilage, calcified cartilage, bone matrix, and calcified bone, these analyses probably describe the changes attending *ossification* and not the fundamental process of calcification. If this is true, the relative increase in Ca and P found in whole bones would not necessarily take place in a microscopic portion of calci-

lying osteoid, the place where bone salt is forming, it is quite possible that the inorganic constituents of bone salt are deposited in constant ratios at the points of calcification or, in other words, the composition of the bone salt is constant during *calcification*. We believe that analyses of enamel by our method eliminate the possibility of these incidental variations in composition, and therefore provide a better description of the process of calcification than serial analyses of growing bones.

From the data obtained here one cannot conclude that the mineral in enamel is an apatite, the atomic ratios (Table I) are not correct for either carbonate apatite ( $\text{Ca P CO}_2 = 10.6:1$ ) or hydroxyapatite ( $\text{Ca P} = 10.6$ ). Nor does the evidence point towards a *variable* mixture of  $\text{Ca}_3(\text{PO}_4)_2$  and  $\text{CaCO}_3$ . On the contrary, the constancy of the Ca P ratio means that Ca is not partitioned between these two components in the varying ratios which have been reported for calcifying bone (13, 14). One may conclude, however, that the calcifying mechanism in enamel of pigs is forming an inorganic substance of constant composition throughout the course of calcification.

#### SUMMARY

In developing pig enamel the Ca, P, and  $\text{CO}_2$  contents were found to increase linearly and in constant ratios to each other throughout the range of calcification, that is, from the lowest to highest degrees of calcification. This constancy is contrasted with the variations in bone found by other workers. It is concluded that these elements are deposited as a complex compound having a fixed composition. Their atomic ratios do not fit the formula for hydroxyapatite or carbonate apatite.

The variable composition of bone usually reported may be the result of histological changes with age rather than a change in the fundamental process of calcification. This suggests that enamel, which is histologically homogeneous and unaffected by growth or resorption, may have advantages over bone for studying the calcification process.

A technique for using the Warburg apparatus to determine  $\text{CO}_2$  in 5 to 10 mg. samples of tissue is described.

The authors are grateful to Mr. George Sheehan of the J. P. Squire Company for making available to us the teeth used in this experiment.

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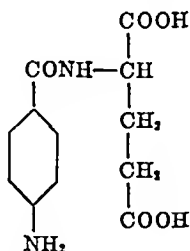
# THE COMPARATIVE ANTISULFANILAMIDE ACTIVITY OF *p*-AMINOBENZOYL-*l*(+)-GLUTAMIC ACID AND *p*-AMINOBENZOIC ACID

By ROGER D WILLIAMS\*

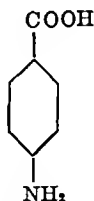
(From the Stamford Research Laboratories, American Cyanamid Company,  
Stamford, Connecticut)

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A number of investigators have directed their efforts towards a search for new and rational approaches to the synthesis of more effective anti-bacterial agents. Some of these efforts have resulted in the preparation and testing of chemical analogues of bacterial growth factors. McIlwain (1) reviews the background of this method of approach and specifically mentions various examples of metabolite antagonism such as that between pantooyltaurine and pantothenic acid, and between pyridine-3-sulfonic acid and nicotinic acid. Another example of the utilization of this method of approach is the investigation by Wooley and White (2) and by Wyss (3) of the antibacterial activity of pyriithamine, the pyridine analogue of thiamine.



*p* Aminobenzoyl-*l*(+)-glutamic acid



*p* Aminobenzoic acid

The present investigation was stimulated by the work of Auhagen (4) who showed that on a molar basis *p*-aminobenzoyl-*l*(+)-glutamic acid (PABG) had an antisulfanilamide activity 8 to 10 times greater than that of *p*-aminobenzoic acid (PAB), when *Streptobacterium plantarum* (10-S) was used as the test organism. The accompanying formulas show the structure of these two analogous compounds.

Our purpose was to determine whether it would be worth while to prepare chemical analogues of PABG for chemotherapeutic investigation. To answer this question we attempted to extend Auhagen's original results to other bacterial species. If, with other species, PABG were found to be

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more active than PAB, it was thought that chemical analogues of PAB might be correspondingly more active than sulfanilamide as chemotherapeutic agents

#### EXPERIMENTAL

The species selected for determining whether Auhagen's findings applied to organisms other than *Streptobacterium plantarum* were *Streptococcus pyogenes*, Group A (C203), *Diplococcus pneumoniae*, Type I (SV-I), *Escherichia coli* (MacLeod), *Clostridium acetobutylicum* (American Type Culture Collection No 862), *Acetobacter suboxydans* (American Type Culture Collection No 621), *Lactobacillus arabinosus* (17-5)

The streptococcus and pneumococcus strains were tested in a peptone-dextrose broth (Bacto-tryptose-phosphate broth) Inocula of approximately 3000 organisms per cc were obtained from young, actively growing cultures of these two organisms

The strain of *Escherichia coli*, an organism which does not require pre-formed PAB for its metabolism, was tested in a synthetic medium, as described by MacLeod (5), consisting of glucose, asparagine, and inorganic salts An inoculum of approximately 700 organisms per cc was used with this organism

*Clostridium acetobutylicum* and *Acetobacter suboxydans* were chosen as representatives of a group of organisms which cannot synthesize PAB and which will grow only when a sufficient amount of PAB is present in the medium The medium, inoculum, and method of culture used for *Clostridium acetobutylicum* were those of Lampen and Peterson (6) The medium, inoculum, and method of culture used for *Acetobacter suboxydans* have been described by Landy and Dicken (7)

We were not able to obtain consistent or satisfactory results with a strain of *Lactobacillus plantarum* in our attempt to repeat Auhagen's original work under conditions similar to those he used This will be discussed later

We were able to approach closely the conditions used by Auhagen by testing *Lactobacillus arabinosus* (17-5) in a semisynthetic medium as described by Snell, Gurard, and Williams (8) All ingredients of this medium were tested for PAB content by the PAB assay method of Lampen and Peterson (6)<sup>1</sup> S M A "vitamin-free" casein hydrolysate was used after it had been treated with charcoal and extracted with ether in order to remove all but slight traces of PAB The PAB removal was carried out as follows 1 gm of Darco S-51 was added to each 100 cc volume of S M A "vitamin-free" casein hydrolysate and stirred mechanically at 60° for 30 minutes The filtrate from this charcoal adsorption still con-

<sup>1</sup> The author is indebted to Dr J O Lampen for the *acetobutylicum* assays

tained a considerable amount of PAB. The filtrate was therefore adjusted to pH 1.8 with concentrated HCl and extracted with ether for 18 to 20 hours in a continuous extractor. The ether layer was separated from the casein hydrolysate and discarded. The casein hydrolysate was readjusted with concentrated NaOH to its original pH of 3.5. At this point it contained only slight traces of PAB which did not interfere with the test.

The same general method of testing was applied to all of the organisms used in this investigation. The determination of the relative antisulfanilamide activity of PAB and PABG was carried out as follows: (1) A suitable medium which would support optimal growth of the organism in question was selected. (2) The minimal concentration of sulfanilamide which would completely inhibit optimal growth was determined by titrating sulfanilamide in a 2-fold dilution series. (3) PAB and PABG were titrated to determine the smallest amount of each required to reverse the inhibition of growth caused by sulfanilamide.

In all instances, the solutions of PAB and PABG<sup>2</sup> were sterilized by filtration through a Seitz EK filter and added aseptically to double strength broth containing the appropriate concentration of sulfanilamide. This method of sterilization was selected in preference to autoclaving in order to prevent the dissociation of PABG into PAB.

With two of the organisms, *Clostridium acetobutylicum* and *Acetobacter suboxydans*, the relative growth stimulation by PAB and PABG was determined by the microbiological assay methods (6, 7) described in the literature for these two organisms.

### Results

The relative antisulfanilamide activity of PAB and PABG was determined for five bacterial species. As shown in Table I, the activity ratio PAB/PABG ranged between 20/1 and 8000/1 on a molar basis. Only with *Lactobacillus arabinosus* was there evidence that PABG, when compared with PAB, had any significant effect in reversing the inhibition of growth caused by sulfanilamide. It is of interest to note that *Lactobacillus arabinosus* is more closely related to *Streptobacterium plantarum*, the organism which Auhagen used in his work, than any of the other species we have tested. With the exception of the experiment in which *Lactobacillus arabinosus* was used, the negligible amount of antisulfanilamide activity obtained with PABG could be accounted for by the presence of as little as 0.1 per cent of PAB as an impurity in the sample of PABG. In the preparation of PABG one might anticipate the formation of traces of PAB

<sup>2</sup> The author is indebted to Dr. Paul H. Bell and Dr. Richard C. Clapp for supplying a sample of *p*-aminobenzoic-(+)-glutamic acid.

due to side reactions, and a chemical purity of greater than 99.9 per cent is not claimed for this compound

A comparison of the growth-stimulating effect of PAB and PABG is shown in Table II. *Clostridium acetobutylicum* and *Acetobacter suboxydans*, both of which are known to require PAB in their metabolism, were selected as test organisms for this part of the investigation. The media in which these two organisms are cultivated can be prepared free of PAB without

TABLE I  
Sulfanilamide Reversal

Organism	Concentration of sulfanilamide used to inhibit growth	Concentration required for reversal of sulfanilamide inhibition			Sulfanilamide reversed by 1 mole of		Activity ratio PAB/PABG (molar basis)
			PAB	PABG	PAB	PABG	
	mg per cent	hrs	mg per cent	mg per cent	moles	moles	
<i>Escherichia coli</i>	10	24	0.0008	13.1	10,000	1.6	6000:1
<i>Clostridium acetobutylicum</i>	5	42	0.001	20.0	4,000	0.5	8000:1
<i>Streptococcus pyogenes</i>	200	16	0.02	16.0	8,000	20.0	400:1
<i>Diplococcus pneumoniae</i> , Type I	200	48	0.008	16.0	20,000	20.0	1000:1
<i>Lactobacillus arabinosus</i>	0.4	24	0.0016	0.064	200	10.0	20:1

PAB = *p*-aminobenzoic acid, PABG = *p*-aminobenzoyl-L(+)-glutamic acid

TABLE II  
Growth Stimulation

Organism	Concentration required to promote optimal growth			Activity ratio PAB/PABG (molar basis)
		PAB	PABG	
	hrs	$\gamma$ per 10 cc	$\gamma$ per 10 cc	
<i>Clostridium acetobutylicum</i>	24	0.0025	10	1500:1
<i>Acetobacter suboxydans</i>	41	0.02	40	750:1

much difficulty (6, 7). As can be seen in Table II, the activity ratios, on a molar basis, are shown to be 1500:1 and 750:1 respectively.

#### DISCUSSION

It is apparent from the data in Tables I and II that Auhagen's results (4) do not apply to the several different types of organisms we have tested. It is of interest to note that Johnson, Green, and Pauli (9) have recently shown that PAB has greater antisulfapyridine activity than PABG with strains of *Escherichia coli*, *Streptococcus hemolyticus*, Group A, and *Diplococcus pneumoniae*, Type III, as the test organisms.

We were not able to repeat Auhagen's original work with *Streptobacterium plantarum*. Although the strain of organism used by Auhagen could not be obtained, an attempt was made to confirm his original work with a similar organism (*Lactobacillus plantarum*, American Type Culture Collection No 4943). With this organism, neither consistent growth nor consistent inhibition of growth by sulfanilamide was obtained with various modifications of test medium and inoculum.

#### SUMMARY

1 *p*-Aminobenzoyl-*l*(+)-glutamic acid, when compared with *p*-aminobenzoic acid, had very little or no antisulfanilamide activity with strains of *Streptococcus pyogenes*, *Diplococcus pneumoniae*, *Escherichia coli*, and *Clostridium acetobutylicum*. With *Lactobacillus arabinosus* (17-5) the anti-sulfanilamide activity of PAB was 20 times greater than that of PABG.

2 The growth-stimulating effect of PABG for *Clostridium acetobutylicum* and *Acetobacter suboxydans* was insignificant when compared with the effect of PAB on these two organisms.

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# INHIBITION OF UTILIZATION OF THIAMINE AND DIPHOSPHOTHIAMINE FOR GROWTH OF MICROORGANISMS\*

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Woolley and White (1) have recently shown that pyrithiamine,<sup>1</sup> the pyridine analogue of thiamine, competitively inhibits the growth of microorganisms requiring thiamine

In the present experiments it was found that less pyrithiamine was required to inhibit growth of *Lactobacillus fermentum* and *Penicillium digitatum* when diphosphothiamine (cocarboxylase) was supplied as a growth factor than when thiamine was used. Since it is generally assumed that the main pathway of thiamine utilization involves preliminary phosphorylation to diphosphothiamine (2-5), and since the organisms studied here also grow faster with diphosphothiamine than with thiamine in media free of inhibitors (6)\* the preliminary findings were investigated further.

This difference in inhibition of thiamine and diphosphothiamine led to examination of many possible factors for explanation, such as permeability, other pathways of thiamine metabolism, or differences in the carboxylase enzyme formed when either thiamine or diphosphothiamine was supplied to the organism. Studies of mixtures of thiamine and diphosphothiamine, effects of other metabolic inhibitors, and correlation of growth and acid production of *Lactobacillus fermentum* suggested a plausible explanation of all of the data, namely, that thiamine must be attached to the carboxylase protein *before* it is phosphorylated, resulting in a carboxylase enzyme different from that obtained when diphosphothiamine is supplied. This agrees with the findings of Westenbrink *et al* (7) on the difference in dissociation of carboxylase normally present in yeast and that formed by adding diphosphothiamine to alkaline washed yeast, and can be correlated with the observations of others on the mechanism of phosphorylation of thiamine to diphosphothiamine (8, 9).

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<sup>1</sup> Pyrithiamine is 1-[(4-amino-2-methyl)-5-pyrimidylmethyl]-2-methyl-3 ( $\beta$ -hydroxyethyl)pyridinium bromide hydrobromide. This compound was kindly given to us by Dr D W Woolley, of The Rockefeller Institute for Medical Research.

<sup>2</sup> Wooster, R C, unpublished observations.



## EXPERIMENTAL

*Methods*

*Lactobacillus fermentum*<sup>3</sup> was grown in the medium used for thiamine assay (6). Varying amounts of thiamine or diphosphothiamine and other substances to be tested (at pH 6.5) were measured into tubes, diluted to 5 ml, and 5 ml of medium added. The tubes were plugged, steamed, and inoculated with a very dilute suspension of a 16 to 24 hour culture of *Lactobacillus fermentum*, as described previously (6). Growth was measured turbidimetrically after 18 to 24 hours and is reported as optical density, which equals log 100 minus log per cent transmission. In some experiments the acid produced in 72 hours was titrated and is given in terms of ml of 0.1 N acid per culture.

*Penicillium digitatum* was grown at pH 4.3, on a medium being used to investigate other growth factors.<sup>4</sup> Tests were grown for 70 hours at 25° in 125 ml Erlenmeyer flasks, and the growth measured as dry weight of the filtered fungus.<sup>4</sup>

*Pyrithiamine Inhibition of Growth of Lactobacillus fermentum and Penicillium digitatum*—Table I shows that for both organisms diphosphothiamine promotes faster growth than thiamine on the plain media employed. However, concentrations of pyrithiamine, which are sufficient to repress growth with diphosphothiamine, are too low to influence growth with equimolecular amounts of thiamine. Ratios of pyrithiamine to thiamine, which partially inhibit growth, completely inhibit response to diphosphothiamine. To show that the differences in inhibition are not due to the use of free thiamine for culturing the organism, experiments with inocula grown with diphosphothiamine, diphosphothiamine and pyrithiamine, or thiamine and pyrithiamine were performed, and the same results obtained.

Using thiamine for growth, Woolley and White (1) found that the inhibition indices (pyrithiamine-thiamine ratios which inhibit growth 50 per cent) could be correlated with the thiamine requirements of the organisms tried. Several, which require the intact thiamine molecule, showed in indices ranging from 7 to 19, whereas, for two others, which require either pyrimidine or thiazole,<sup>5</sup> the indices were increased to 130 and 800, respectively. From the data of Table I the inhibition index for *Lactobacillus*

<sup>3</sup> While this paper was in press, Dr. J. M. Sherman of Cornell University suggested that the preferred name for this organism is *Lactobacillus fermenti*.

<sup>4</sup> The authors wish to thank Miss Ruth C. Wooster for carrying out the experiments with *Penicillium digitatum*.

<sup>5</sup> The pyrimidine and thiazole moieties of the thiamine molecule refer to 2-methyl-5-ethoxymethyl-6-aminopyrimidine and 4-methyl-5-hydroxyethylthiazole, respectively.

*fermentum* is about 50 when the organism is grown with thiamine and only 10 with diphosphothiamine. Since this organism can utilize thiazole about 5 per cent as well as thiamine in 18 hours (6), the index of 50 falls roughly into the pattern set up by Woolley and White (1). For *Penicillium digitatum*, which requires only the thiazole moiety for growth, the data of Table I are not sufficiently complete for accurate calculation of inhibition indices, but indicate indices of about 150 with thiamine and 40 with diphosphothiamine.

TABLE I

*Inhibition by Pyrithiamine of Utilization of Thiamine and Diphosphothiamine for Growth of Lactobacillus fermentum and Penicillium digitatum*

Amounts of thiamine and diphosphothiamine are given in terms of thiamine hydrochloride for comparison on a molecular basis.

The figures for *L. fermentum* represent turbidimetric measurements of growth after 18 hours, and are given in terms of optical density (log 100 minus log per cent transmission). The results for *P. digitatum* are presented in mg. as dry weight of the fungus after 70 hours of incubation.

Organism	Pyrithiamine	Thiamine					Diphosphothiamine			
		0.00 $\gamma$	0.01 $\gamma$	0.02 $\gamma$	0.03 $\gamma$	0.04 $\gamma$	0.01 $\gamma$	0.02 $\gamma$	0.03 $\gamma$	0.04 $\gamma$
<i>L. fermentum</i>	$\gamma$									
	0.0	0.040	0.220	0.370	0.460	0.560	0.250	0.400	0.490	0.580
	0.1	0.025	0.230	0.380	0.470	0.550	0.175	0.390	0.480	0.570
	0.3	0.010	0.220	0.390	0.460	0.570	0.010	0.115	0.340	0.440
	1.0	0.005	0.010	0.250	0.430	0.550	0.005	0.010	0.010	0.005
<i>P. digitatum</i>		0.0 $\gamma$	0.25 $\gamma$	1.0 $\gamma$			0.25 $\gamma$	1.0 $\gamma$		
	0	8	33	50			41	53		
	40	2	20	45			13	50		
	100	2	14	43			4	16		

*Inhibition of Mixtures of Thiamine and Diphosphothiamine*—Since derivatives of thiamine containing the 6-aminopyrimidine group inhibit the splitting of diphosphothiamine to thiamine by phosphatases (10-12), it was assumed that pyrithiamine could also repress this hydrolysis and prevent the formation of free thiamine. If a small amount of free thiamine were needed for growth of the organisms, this might then explain the difference in repression by pyrithiamine of thiamine and diphosphothiamine activity. However, this possibility seemed unlikely, since the amounts of pyrithiamine employed were not considered enough to inhibit completely the breakdown of diphosphothiamine (10-12). The following experiments on the inhibition of mixtures of thiamine and diphosphothiamine rendered the possibility even less plausible. Table II shows

that replacement of part of the diphosphothiamine by thiamine increases the inhibition indices over those obtained with only diphosphothiamine, but not to the levels observed with thiamine alone. If the inhibitions of diphosphothiamine were caused by a lack of some free thiamine, the mixtures should respond similarly to thiamine in their inhibition by pyriithiamine. The partial increases in inhibition indices which are observed are evidently due to the growth with the thiamine present. This was shown further for *Lactobacillus fermentum* by addition of sterile pyriithiamine solution to cultures in which growth had just become visible after 10 to 12 hours of incubation. The turbidity readings after 18 hours growth showed that the amounts of pyriithiamine necessary for inhibition

TABLE II

*Inhibition by Pyriithiamine of Utilization of Mixtures of Thiamine and Diphosphothiamine for Growth of Lactobacillus fermentum and Penicillium digitatum*

Organism	Pyriithiamine	Thiamine and diphosphothiamine equal amounts*				
		0.00 $\gamma$	0.005 $\gamma$	0.01 $\gamma$	0.015 $\gamma$	0.02 $\gamma$
<i>L. fermentum</i>	$\gamma$					
	0.0	0.040	0.270	0.410	0.550	0.640
	0.3	0.020	0.180	0.360	0.540	0.650
	1.0	0.015	0.010	0.015	0.195	0.450
<i>P. digitatum</i>		0.0 $\gamma$	0.125 $\gamma$	0.25 $\gamma$	0.5 $\gamma$	
	0	8	32	48	51	
	40	2	17	35	45	
	100	2	9	19	43	

\* See the note above Table I

under these conditions were much larger than when the pyriithiamine was added with the medium. In these experiments also, pyriithiamine inhibition was greater for diphosphothiamine than for thiamine.

*Effects of Other Metabolic Inhibitors on Growth and Acid Production of Lactobacillus fermentum*—Although the main pathway of thiamine metabolism apparently proceeds through the carboxylase enzymes, it seemed possible that alternate routes may be more important in the presence of some inhibitors (13).

In order to determine whether the presence of pyriithiamine, in concentrations sufficient for inhibition of diphosphothiamine, caused thiamine to assume another metabolic pathway, the acid produced by *Lactobacillus fermentum* in the presence of thiamine and pyriithiamine was compared to that formed with thiamine alone. Table III shows that there were no changes in acid production in this case. The effect of pyriithiamine

on the acid production with monophosphothiamine and with diphosphothiamine, as contrasted with the growth in 24 hours, shows that in 3 days the organism was able to overcome some of the inhibition. Monophosphothiamine was prepared from diphosphothiamine by heating at 100° for 15 minutes in 1 N HCl (2). The inhibition index for *Lactobacillus fermentum* with the monophosphate is slightly higher than that obtained with diphosphothiamine, but far short of the thiamine figure.

TABLE III

*Effect of Several Inhibitors upon Utilization of Thiamine, Monophosphothiamine, and Diphosphothiamine for Growth and Acid Production of Lactobacillus fermentum*

Inhibitor	Thiamine*			Monophosphothiamine*		Diphosphothiamine*	
	0.00 $\gamma$	0.02 $\gamma$	0.2 $\gamma$	0.02 $\gamma$	0.2 $\gamma$	0.02 $\gamma$	0.2 $\gamma$
Turbidimetric readings, 24 hrs. growth							
None	0.100	0.700	1.3	0.720	1.3	0.750	1.3
Pyrithiamine, 1 $\gamma$ per tube	0.000	0.420	1.3	0.000	1.2	0.000	1.1
Fluoride, 0.04 M	0.075	0.275	0.315	0.260	0.315	0.275	0.340
Iodoacetate, 0.0001 M	0.060	0.670	1.2	0.640	1.2	0.650	1.2
2,4-Dinitrophenol, 0.001 M	0.040	0.420	0.700	0.390	0.650	0.400	0.630
Malonate, 0.1 M	0.050	0.220	0.375	0.200	0.340	0.210	0.350
Cyanide, 0.02 M	0.030	0.260	0.320	0.250	0.290	0.260	0.280
0.1 N acid produced, 72 hrs. ml							
None	3.0	5.2	8.6	5.1	8.6	5.1	8.4
Pyrithiamine, 1 $\gamma$ per tube	0.9	4.9	8.5	4.1	8.4	3.5	8.2
Fluoride, 0.004 M	0.4	0.5	0.9	0.4	0.8	0.4	0.8
Iodoacetate, 0.0001 M	1.4	2.3	4.1	2.1	4.0	2.3	4.3
2,4-Dinitrophenol, 0.001 M	2.5	3.7	8.2	3.8	8.4	3.7	8.4
Malonate, 0.1 M	2.8	3.6	6.8	4.0	6.9	3.9	7.1
Cyanide, 0.02 M	2.9	5.4	7.5	5.2	7.4	5.5	7.6

\* See the note above Table I

The growth and acid production in the presence of other known inhibitors of intermediate processes in carbohydrate metabolism were tried to determine whether other metabolic pathways exist for *Lactobacillus fermentum* and, if so, whether they involved differences in the form of thiamine supplied. The growth in the presence of fluoride or iodoacetate as contrasted with the very low acid production shows that alternate growth mechanisms do exist (Table III).

Iodoacetate, which in a concentration of 0.0009 M inhibits synthesis of diphosphothiamine (4, 14) but which has no effect on the action of carboxylase (14, 15), has no effect on growth of *Lactobacillus fermentum*.

(at 0.0001 M), but severely curtails acid production (Table III). At higher concentrations of iodoacetate, growth with thiamine and with diphosphothiamine is inhibited equally.

At a level of 0.04 M, fluoride does not markedly inhibit synthesis of diphosphothiamine (4, 14). At this concentration, fluoride permits some growth of *Lactobacillus fermentum*, but not through a pathway involving acid production. Similarly, Wiggert and Werkman (13) showed that propionic acid bacteria can also metabolize glucose through another pathway. Those grown in the presence of glucose and a concentration of fluoride which inhibited the breakdown of phosphoglyceric acid were able to utilize glucose but not phosphoglyceric acid.

The other inhibitors affect growth and acid production as shown in Table III. 2,4-Dinitrophenol has been shown to retard pyruvic acid oxidation by brain tissue (16). Malonic acid inhibits the C<sub>4</sub>-dicarboxylic acid systems which are necessary to pyruvate metabolism (17, 18), and cyanide has been shown to inhibit the oxidation of pyruvic acid by gonococci (19). At the concentrations used, these three inhibitors have little effect on the 72 hour acid production, but repress the 24 hour growth.

Since each of the above inhibitors affects the synthesis or activity of diphosphothiamine, and shows the same effect on growth and acid production with all three forms of thiamine, it seems unlikely that thiamine is involved differently from its phosphate esters in the alternate metabolic pathways.

*Effect of Pyrimidine Compounds on Utilization of Thiamine and Diphosphothiamine by Lactobacillus fermentum*—In order to learn whether the mechanisms for conversion of added thiamine or diphosphothiamine to carboxylase enzymes differed, 6-aminopyrimidine compounds were used to prevent completely any hydrolysis of diphosphothiamine to thiamine (11, 12). Westenbrink *et al.* (12) calculated, from the relative stimulation of carboxylase activity by thiamine and aminopyrimidines (10, 11), that the presence of 150 to 200  $\gamma$  of the pyrimidine compound would entirely prevent the splitting of 1  $\gamma$  of diphosphothiamine to thiamine. The pyrimidines used in our experiments were present in amounts up to almost 10,000 times the weight of diphosphothiamine present. The effects of 2-methyl-6-aminopyrimidine, 2-methyl-5-ethoxymethyl-6-aminopyrimidine, and 2,6-dioxypyrimidine (uracil) on the growth of *L. fermentum* with thiamine, monophosphothiamine, and diphosphothiamine were tested. 2-Methyl-5-ethoxymethyl-6-aminopyrimidine is the pyrimidine moiety of thiamine and cannot be utilized by *L. fermentum* to replace thiamine for growth under the conditions employed. The results with the three pyrimidines are shown in Table IV.

Uracil is a 6-oxypyrimidine which, at a level of 200  $\gamma$  per tube, has no

effect on growth with all three forms of thiamine. However, 100  $\gamma$  of the 6-aminopyrimidine compounds inhibit growth with diphosphothiamine and with monophosphothiamine, but have no effect upon the response to thiamine. In the presence of 200  $\gamma$ , the 18 hour growth with thiamine is inhibited slightly (Table IV). After 72 hours, the organism is able to overcome these inhibitions and produces almost as much acid as those grown without aminopyrimidines.

This effect of 6-aminopyrimidines upon the utilization of diphosphothiamine is opposite to the stimulation of yeast carboxylase by relatively smaller amounts of these substances (10-12). Aminopyrimidines are about one-tenth as active as thiamine in preventing the enzymatic splitting of diphosphothiamine by phosphatases (10-13). The inhibition of ac-

TABLE IV

*Effect of Pyrimidine Compounds upon Utilization of Thiamine, Monophosphothiamine, and Diphosphothiamine for Growth of Lactobacillus fermentum in 18 Hours*

Substance added	Per tube	Thiamine			Monophosphothiamine*		Diphosphothiamine*	
		0.00 $\gamma$	0.01 $\gamma$	0.02 $\gamma$	0.01 $\gamma$	0.02 $\gamma$	0.01 $\gamma$	0.02 $\gamma$
None	$\gamma$							
2-Methyl-5-ethoxymethyl-6-aminopyrimidine		0.025	0.220	0.340	0.220	0.330	0.230	0.360
"	100	0.010	0.240	0.350	0.025	0.065	0.015	0.035
"	200	0.010	0.130	0.295	0.005	0.015	0.005	0.000
2-Methyl-6-aminopyrimidine	100	0.015	0.225	0.350	0.070	0.220	0.040	0.150
2,6-Dioxypyrimidine (uracil)	200	0.040	0.235	0.330	0.240	0.340	0.240	0.350

\* See the note above Table I

tivity of added diphosphothiamine by *excess* pyrimidine is competitive, and that of thiamine at much higher concentrations is also competitive. This indicates that the dissociation of the thiamine-protein complex is much less than that formed by diphosphothiamine and protein, and agrees with the findings of Westenbrink *et al.* (7) on the dissociation of naturally occurring carboxylase and of that formed by the addition of diphosphothiamine to alkaline washed yeast.

#### DISCUSSION

Pyrimthiamine competitively inhibits reactions in which thiamine or diphosphothiamine participates. Since diphosphothiamine is the main form in which thiamine is known to function (2-5), and since thiamine must be phosphorylated before use in enzyme systems, it was expected that pyrimthiamine would inhibit growth with thiamine more than with

diphosphothiamine The opposite effect found here suggested several explanations

The more rapid growth with diphosphothiamine than with thiamine in media free of inhibitors precluded any important difference in permeability of the two substances, such as is found with brain tissue (17) However, since we were measuring growth and not carboxylase activity, the possibility also existed that some free thiamine was necessary for other functions of the microorganisms The inhibition of mixtures of thiamine and diphosphothiamine showed that this was not the case Experiments with other metabolic inhibitors, such as iodoacetate, fluoride, cyanide, dinitrophenol, and malonate, demonstrated that, although other metabolic pathways for growth exist in *Lactobacillus fermentum*, the same effects on growth and acid production occur if thiamine, monophosphothiamine, or diphosphothiamine is present This indicates that all three forms of thiamine are probably being used in the same manner

The hypothesis that the formation of carboxylase enzyme from thiamine differs from that formed with diphosphothiamine, and results in enzymes similar in activity but differing in dissociation, seems to offer the best explanation of the data presented These conclusions are based mainly on the experiments with pyrithiamine and with 6-aminopyrimidines, and are supported by the findings of Westenbrink *et al* (7)

Although Buchman *et al* (20) and Stern and Melnick (21) have presented good evidence for the attachment of preformed diphosphothiamine to the carboxylase protein through the pyrophosphate group, Westenbrink *et al* (7) have shown that this is a highly dissociated linkage, unlike the tight bond found in naturally occurring carboxylase Green and coworkers also showed that carboxylase isolated from yeast is not readily dissociable (15) Westenbrink *et al* (7) postulated two linkages for the non-dissociated enzyme, one through the pyrimidine amino group to a carboxyl group of the protein (tightly bound), and the other a salt-like linkage of the pyrophosphate group and an amino group of the protein (highly dissociated) Diphosphothiamine added to the apoenzyme forms only the latter linkage

In the present experiments, the inhibition of diphosphothiamine by pyrithiamine and aminopyrimidines can be explained by their ability to form strongly bound linkages to the enzyme protein, displacing diphosphothiamine from its position on the protein molecule Thiamine can not only compete readily with pyrithiamine or aminopyrimidines for this attachment, but forms stronger linkages than these substances (1, 10-12) These findings indicate that free diphosphothiamine is not a normal intermediate in the utilization of thiamine, and suggest that thiamine forms a relatively undissociable linkage to protein before it is phosphorylated

If thiamine were converted to diphosphothiamine before being attached to protein, it would be inhibited by pyrithiamine and aminopyrimidines to an equal or greater degree than is observed for diphosphothiamine

The existence of thiamine-protein complexes in many natural materials (22, 23) and the ability of tissues to synthesize only enough diphosphothiamine to saturate, partly or fully, the protein moiety of the enzyme (8, 9) sustain these conclusions. Other findings which lend support are the lack of inhibition of growth of *Lactobacillus fermentum* by pyrimidines lacking the 6 amino group (uracil) and the inability of the 6-hydroxy derivative of thiamine to replace thiamine as a vitamin (24)

#### SUMMARY

Pyrithiamine, the pyrimidine analogue of thiamine, and 6-aminopyrimidine compounds inhibit the utilization of diphosphothiamine for growth of *Lactobacillus fermentum* more than they inhibit the use of thiamine. The presence of some free thiamine does not overcome this difference in inhibition. The findings with pyrithiamine are the same for growth of *Penicillium digitatum*.

Addition of iodoacetate, fluoride, malonate, cyanide, or dinitrophenol inhibits growth or acid production of *Lactobacillus fermentum*. The effects are the same in the presence of thiamine, monophosphothiamine, or diphosphothiamine.

The results indicate that carboxylase formed from thiamine is a more firmly bound enzyme than that formed from added diphosphothiamine, and suggest that thiamine is attached to the apoenzyme before being phosphorylated.

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# THE CHEMISTRY OF PHYTOMONAS TUMEFACIENS\*

## IV CONCERNING THE STRUCTURE OF PHYTOMONIC ACID

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(Received for publication, June 29, 1944)

The isolation and purification of phytomonic acid from the phosphatides of the pathogenic plant bacillus, *Phytomonas tumefaciens*, have recently been described (1). On the basis of its empirical composition ( $C_{20}H_{40}O_2$ ), its low melting point ( $24^\circ$ ), and the high solubility of its lead salt in ether a saturated branched chain structure was proposed. Because of the unfavorable properties of the acid a structure study by degradative procedures on the few hundred mg. of available material did not seem promising. It has been possible, however, to obtain a certain amount of information concerning the general features of the structure by x-ray diffraction methods.

The fragmentary crystals of phytomonic acid, obtained by slow cooling of the melt, resemble those of the normal fatty acids, occurring in the form of thin leaflets with cleavage parallel to the leaflet surface. Although not suitable for single crystal analysis, the soft leaflets could be oriented in one direction by gently pressing them on a glass plate. Nickel-filtered copper radiation was employed and the diffraction patterns were recorded on a cylindrical camera of 51.2 mm. radius. Oscillation of the preparations over a range of  $0$  to  $20^\circ$  occurred at constant angular velocity. The measurements have an estimated accuracy of about  $\pm 1$  per cent.

The x-ray reflections obtained at small angles of incidence from the leaflet surface contained lines up to the twentieth order of a spacing of 40.5 Å. Long crystal spacings obtained in this fashion are a characteristic of the simpler long chain compounds (2). They arise from the fact that the molecules are oriented approximately lengthwise between planes which are parallel to the leaflet surface. The observed spacing is equivalent to that of a normal chain of about 30 carbon atoms oriented perpendicularly to the reflecting planes. Since phytomonic acid contains only 20 carbon atoms the spacing must correspond as in the series of normal acids to a double layer of molecules. Accordingly the minimum chain length of phytomonic acid, if the molecules have the perpendicular orientation, is 14 or 15 carbon atoms. A perpendicular orientation, however, has

\* This work was aided by a grant from The International Cancer Research Foundation.

never been observed in crystals of the fatty acid series and it would appear that an inclined orientation is a requirement for the packing of the carboxyl group. Phytomonic acid would therefore be expected to have a chain length somewhat longer than 15 carbon atoms.

TABLE I

*Observed Crystal Spacings of Some Branched Chain Acids, Amides, and Hydrazides*

Compound	Long spacing	Side spacings		
		A	A	A
<i>dl</i> -2-Methylstearic acid*	37.4	3.74	4.03	
<i>dl</i> -15-Methylstearic " †	35.8	3.62		
<i>dl</i> -16-Methylstearic " ‡	28.0	3.74		
<i>dl</i> -17-Methylstearic " §	36.2	3.79	4.15	
Phytomonic acid	40.5	3.67	4.10	4.59
<i>dl</i> -2-Methyleicosanoic acid*	40.4	3.75	4.07	
<i>dl</i> -10-Methylhexacosanoic acid*	53.7	3.69	4.15	
Stearamide	36.2	3.77	4.16	
Tuberculostearamide	37.8	3.79	4.10	
<i>dl</i> -10-Methylstearamide¶	39.1			
<i>dl</i> -15-Methylstearamide†	36.7	3.76	4.19	4.54
3-Methylstearamide‡	35.8	3.82	4.16	
7-Methylstearamide§	36.9	3.67		
2-Methylstearic hydrazide**	37.5			
Phytomonic hydrazide**	38.3	3.67	4.10	4.55
Stearic hydrazide**	52.0			
Myristic " **	42.3			
Lauric " **	36.9			
Stearic acid	39.7	3.69	4.04	

\* Samples of these acids, synthesized by Schneider and Spielman (3), were provided by Dr. M. A. Spielman.

† Sample provided by Dr. James Cason (13).

‡ Sample provided by Dr. James Cason, synthesized by Cason and Prout (4).

§ Sample of acid synthesized by Dr. James Cason (5).

|| Prepared from a sample of tuberculostearic acid isolated by Dr. R. J. Anderson from the lipids of the tubercle bacillus (6). This is believed to be the optically active form of 10-methylstearic acid (7).

¶ Prepared from a sample of *dl*-10-methylstearic acid synthesized by Dr. M. A. Spielman (8).

\*\* The hydrazides were prepared from the methyl esters by refluxing with an excess of hydrazine hydrate. They were recrystallized from methanol.

In the absence of more complete crystallographic data it was not possible to determine the angle of inclination of the molecules and hence to fix the chain length directly. For purposes of comparison diffraction photographs were taken of a number of synthetic branched chain acids containing secondary methyl groups. The observed crystal spacings of these acids

and some of their derivatives are listed in Table I. In general the branched acids, although apparently crystallizing in the same general form, give spacings somewhat shorter than do the normal acids containing the same number of chain carbon atoms. The spacings are seen to vary to a certain extent with the position of the branch on the chain as well as with the number of chain carbon atoms. Similar results have been obtained by Stenhagen on multilayers deposited from surface films (9).

The amides give spacings differing only slightly from those of the free acids. Although 16-methylstearic acid gives an abnormally short spacing, its amide falls in the usual range. No evidence has so far been obtained of polymorphism in preparations crystallized from various solvents or directly from the melt. The spacings of the normal hydrazides are of interest since they likewise assume the double layer structure but give spacings corresponding to an approximately vertical orientation of the molecules. In the branched series, however, the hydrazides are not necessarily vertically oriented, as can be seen from the spacings of the hydrazides of phytomonic acid and 2-methylstearic acid.

The number of end methyl groups attached to carbon in phytomonic acid was determined by the chromic acid oxidation procedure of Kuhn and Roth (10). A sample weighing 14.95 mg yielded on oxidation and distillation 1.64 molecules of acetic acid per molecule<sup>1</sup>. This was a little less than twice the amount of acetic acid given by a series of normal acids (0.8 to 0.9 molecules per molecule) and approximately equal to the amounts obtained from samples of 2-, 10-, 15-, and 16-methylstearic acids. Phytomonic acid therefore contains two end methyl groups attached to carbon and hence a single side chain.

The side spacings listed in Table I cannot be indexed with certainty but have been shown in the normal series to correspond to lateral spacings of the long chains. It is to be observed that some of the branched compounds give spacings very close to those obtained from compounds with normal chains. The presence of a secondary methyl group therefore does not necessarily cause a marked increase in the side spacings, and it would appear that a secondary methyl group may at least in certain cases be accommodated in the normal lattice with relatively little distortion. The phytomonic acid leaflets showed some tendency toward a preferred orientation with the side spacing arcs (which because of the size of the crystallites were spotty) increasing in intensity in the direction of a layer line. The values obtained fall in the range of those given by the methyl-substituted acids and their derivatives. A methyl side chain is therefore possible.

The presence of branching in phytomonic acid is clearly demonstrated by the intensity distribution of its long spacing reflections which differs in

<sup>1</sup> These determinations were made by Dr. L. G. Ginger.

a characteristic fashion from those of the normal acids. The intensity distribution of these reflections is determined chiefly by the distribution of electron density along a line normal to the reflecting planes. It is therefore a function of the distribution of scattering power along the molecular axis and is sensitive to the size, number, and position of the substituent groups as well as to the arrangement of the molecules in the crystal. The effect was first demonstrated on several series of normal aliphatic compounds by Shearer (11) and has been employed by Chibnall, Piper, Pollard, Smith, and Williams (12) in the identification of long chain ketones isolated from the wax of apple cuticle.

In Fig. 1 the relative intensity distributions of a number of acids and their derivatives are shown. It should be noted that the patterns of the two normal homologues (palmitic and stearic acid) differing by 2 carbon atoms in chain length show a very gradual shift in the sequence with no abrupt change in the general pattern. This effect is also observed in the patterns of 2-methylstearic and 2-methyleicosanoic acids and in the sequence of the normal hydrazides. In the latter series the acid molecules have in effect been lengthened by 1 atom at the carboxyl end. The large deviation in their patterns from those of the normal acids arises not only from the chain lengthening but also from the fact that the hydrazides are approximately vertically oriented whereas the acids are tilted.

The introduction of a secondary methyl group causes a marked alteration of the pattern which is furthermore influenced by the position of the group. The available methylstearic acids represent the extremes of the series with branches near either end of the molecule and in the middle. Due to its low melting point a detailed pattern could not be obtained from 10-methylstearic acid but a good one was obtained from its higher melting amide (7). The amides of the 15- and 17-methyl acids give patterns resembling those of the free acids. Since the amide group has approximately the same scattering power as the hydroxyl group which it replaces, the observed agreement indicates a very similar arrangement of the molecules in the crystal. On the other hand the intensity distributions of the 16-methyl acid and its amide deviate more widely, which was to be expected since there is a great difference in their long spacings.

The patterns obtained from the 15-methyl acid and from its amide (not shown) exhibit a rapid decline in intensity in the higher orders. Results of this type are usually obtained from crystals containing homologous or isomeric impurity. In this respect the large number of reflection orders obtained from phytomonic acid and its hydrazide is significant since in the purification of phytomonic acid the possible presence of isomers capable of forming mixed crystals had not been definitely excluded.

The diffraction pattern given by phytomonic acid resembles those of both

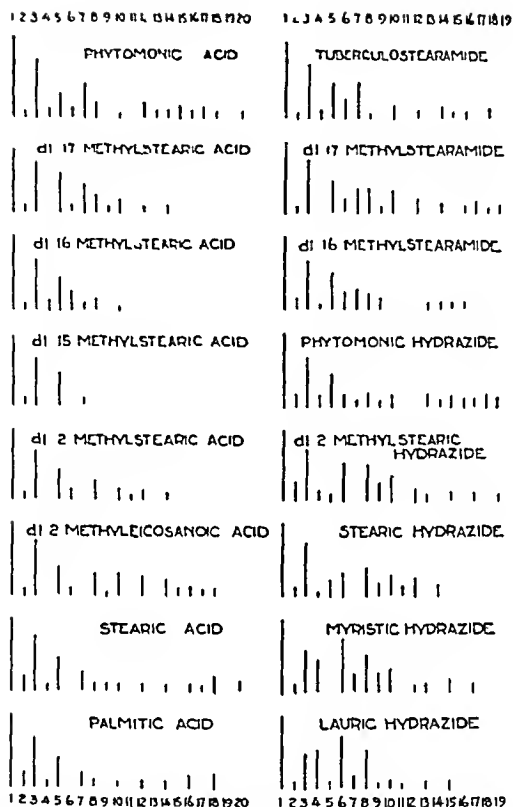


FIG 1 Diagrams of intensities of long spacing reflections The intensity of the  $l$ th order reflection for the planes under consideration is given by the expression

$$I_l = K \frac{1 + \cos^2 2\theta}{\sin 2\theta} \left[ \sum_n f_n \cos 2\pi l z_n \right]^2$$

where  $z_n$  is the coordinate of the  $n$ th chain atom,  $f_n$  its scattering coefficient, and  $\theta$  the Bragg angle. It was shown in a previous report (7) that a set of atomic coordinates,  $z_n$ , corresponding to the accepted planar zigzag structure of stearic acid tilted in the direction of its broader face gives, when substituted in the above equation, a set of relative intensities in good agreement with the experimental values. The introduction in this simplified model of an additional scattering term at the level of the 10th carbon atom gave a series of calculated relative intensities in fair qualitative agreement with those obtained from the amide of tuberculostearic acid. When a similar procedure is applied to the acids discussed in the present report, the agreement with the experimental values is rather poor. The crystals of the latter acids are thus not sufficiently isomorphous with stearic acid to justify the use of a common set of chain parameters.

the branched and normal acids in that the early odd orders are strong and the even orders weak. This supports the double layer crystal structure proposed on the basis of the long spacings. The long spacing, moreover, is sufficiently larger than those of the methyl-substituted stearic acids to be compatible with a chain length of 19 carbon atoms. This would leave 1 carbon atom available for the side chain which would accordingly be a methyl group as previously suggested from the side spacings. If the side chain is a methyl group it must necessarily be located near the middle of the chain. This requirement is imposed by the fact that phytomonic acid melts more than  $50^\circ$  below its normal isomer (*n*-eicosanoic acid, m p  $76.4^\circ$ ) and forms a lead salt easily soluble in ether. The melting points of the methyl-substituted acids decrease as the methyl group is moved inward from either end of the molecule toward the center. Thus of the members of the methylstearic acid series so far synthesized *dl*-10-methylstearic acid (m p  $20^\circ$ ) has the lowest melting point ( $49^\circ$  lower than stearic acid). Similarly it is the only member so far observed to form an ether-soluble lead salt.

In view of the above considerations it is of interest to continue the comparison of the intensities. It should be noted that such a comparison involves the assumption that the long chain portions of the molecules contribute in a similar fashion to the scattering of the x-rays. This has been shown to be approximately true in the case of tuberculostearamide and stearic acid but to hold less rigorously for the acids containing secondary methyl groups near either end of the chain. Aside from the previously mentioned similarity the phytomonic acid pattern differs markedly in the early orders from those of the terminally substituted structures. This was to be expected in view of the differences in the other physical properties and may be attributed both to structural and crystallographic differences. Of the patterns available for comparison that of tuberculostearamide, which has a methyl group in the 10 position, resembles most closely that of phytomonic acid. In particular the decline in intensity of the early odd orders is interrupted by a strong seventh order, and the weak even orders which usually show a preliminary decline reach an early maximum at the sixth and eighth orders respectively. In both cases the ninth and eleventh orders are weak. Quantitative differences occur in all orders, particularly in the higher ones. These differences are of less significance insofar as the grosser features of the structure are concerned and may be due in part to accidental properties of the particular oriented crystal aggregate being studied.

The demonstration of purity of naturally occurring acids of high molecular weight isolated from complex mixtures of isomers and homologues has always been a difficult one, due to the possibilities of mixed crystal formation and the small divergence in physical properties. The present

results indicate with a considerable degree of certainty that phytomonic acid is a single entity in a relatively high state of purity. Moreover, there is little doubt that the acid possesses a relatively long chain structure and a single side chain, necessarily a small one. The problem of uniquely determining the size and position of the side chain has not been completely solved. That the side chain is a methyl group appears probable, in which case it must, from the properties of the acid, be located near the middle of the chain. The present x-ray technique, although providing a great deal of information from a minimum amount of material, is limited by the nature of the crystals available. A comparison with similar but not identical structures narrows the field of possibilities and may, as in the present case, when interpreted with respect to consistency with data from other sources, provide a provisional structure. Such a structure, consistent with the observed facts, is 10- or 11-methylnonadecanoic acid.

#### SUMMARY

1 Crystal spacings and relative intensities of the long spacing x-ray reflections have been determined for phytomonic acid and a number of synthetic branched chain acids and derivatives.

2 The purity of phytomonic acid has been established and it has been shown to have a relatively long chain structure and a single small side chain, probably a methyl group.

3 A provisional structure for phytomonic acid, consistent with the available data, is discussed.

This work is part of a general study of bacterial lipids being conducted in this laboratory by Professor R. J. Anderson whose encouragement I gratefully acknowledge. The x-ray facilities were made available by Dr. George Switzer of the Department of Mineralogy.

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# TRIOSE PHOSPHATE ISOMERASE\*

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Triose phosphate isomerase (or more briefly isomerase) is an enzyme discovered in 1934 (1, 2) which catalyzes the reaction glyceraldehyde phosphate  $\rightleftharpoons$  dihydroxyacetone phosphate. The transformation of hexose diphosphate into triose phosphate is brought about by the joint action of two enzymes, called together zymohexase, namely, aldolase, which splits the hexose ester into 1 glyceraldehyde phosphate + 1 dihydroxyacetone phosphate, and isomerase (3). In the absence of isomerase, living cells would be able to metabolize only *half* of the hexose molecule by way of glyceraldehyde phosphate to pyruvic acid and its various end-products (lactic acid in muscle, alcohol and CO<sub>2</sub> in yeast, etc.)

Separation and purification of isomerase became important in connection with the study of the equilibrium between glyceraldehyde phosphate and its oxidation product in the presence of cozymase (4). Moreover, it seemed of interest to test the claim of Iri (5) that this isomerase is identical with hexose phosphate isomerase, formerly called phosphohexomutase. According to Lohmann (6) hexose phosphate isomerase transforms glucose-6-phosphate and fructose-6-phosphate into an equilibrium mixture of the two esters. If Iri's claim were true, the enzyme would exhibit a class specificity for aldose-ketose transformation. The claim was based by Iri on the observation that crude muscle extracts containing hexose phosphate isomerase and somewhat purified fractions were able to establish the equilibrium of the triose phosphates.

## Methods

The activity of the isomerase is measured by the speed of transformation of glyceraldehyde phosphate into dihydroxyacetone phosphate by given amounts of protein which contain the enzyme. Since the reaction leads to an equilibrium at about 4 per cent glyceraldehyde phosphate (4) and roughly follows, up to about 50 per cent turnover, the kinetics of a monomolecular equilibrium reaction, only the first part of the curve can be used for the computation of the rate of glyceraldehyde phosphate trans-

\* The work was aided by grants to O. Meyerhof from Hoffmann-La Roche, Inc., Nutley, New Jersey, and the American Philosophical Society.

formation, without cumbersome calculations of velocity constants. Moreover, the preparations of *d*-glyceraldehyde phosphate, made by way of the hydrazone (4), already contain between 20 and 40 per cent dihydroxyacetone phosphate. Therefore it is preferable to employ amounts of enzyme which do not bring about transformation of more than about a third of the initial glyceraldehyde phosphate in the chosen time interval. Measurements were made at 37° for 3 or 4 minutes.

The methods for preparing triose phosphate and for determining small amounts of glyceraldehyde phosphate in the presence of variable amounts of dihydroxyacetone phosphate have already been described (4). For a great part of the experiments the simpler method was used, consisting in the measurement of the alkali-labile P before and after treatment with iodine in weakly alkaline solution. The results were checked by similar experiments, in which the more accurate method of optical rotation of phosphoglyceric acid in molybdate was used as a measure for the glyceraldehyde phosphate present. This method requires much more time and greater amounts of triose phosphate. The accuracy of both methods is limited by the difficulty of securing complete oxidation of the aldotriose without attacking the ketotriose. Occasional failures are therefore inevitable, so that whenever possible each activity value given represents the mean of two or more single experimental values.

A simple means for comparing the activity of different preparations as well as of different enzymes which act on phosphorylated compounds is to express all data in mg of P metabolized in 1 minute at 38°, and call 1 unit of enzyme the amount which transforms 1 mg of P in 1 minute. "Turn-over numbers," based on the (arbitrary) assumption of a molecular weight of 100,000 for this and similar enzyme proteins, are 3200 times these activity values.

In many of the earlier experiments the dry weight of materials precipitated by trichloroacetic acid was used as a measure of the amount of protein. The trichloroacetic acid precipitates were caught on filter paper circles, washed with about 20 cc of 1 per cent trichloroacetic acid to remove salts, particularly ammonium sulfate, and dried at 105° to constant weight.

As the purification process proceeded, the amount of enzyme material available became insufficient for accurate dry weight determinations. Protein was then estimated from N values obtained by micro-Kjeldahl determination plus direct nesslerization, with gum ghatti as a protective colloid. In the N determinations it was first necessary to remove the ammonia present as ammonium sulfate by washing the trichloroacetic acid precipitate with dilute trichloroacetic acid in the centrifuge tubes. Ratios of dry weight to N, instead of approaching a value of 6.25, ranged from about 7.5 to 9.

The amounts of ammonium sulfate precipitated by trichloroacetic acid

were found not to have any effect on protein values determined by the biuret method, as described by Robinson and Hogden (7). This method gave values for protein in enzyme preparations somewhat less than those obtained from the dry weight determinations, and somewhat greater than those obtained from the N determinations. Casein solutions were used as standards for the amount of protein in the N and biuret determinations.

*Separation of Isomerase from Aldolase*—In earlier work (8) it was shown that when triose phosphate was trapped by means of hydrazine or sulfite hexose diphosphate was split enzymatically into a triose phosphate mixture containing over 50 per cent glyceraldehyde phosphate, instead of the usual equilibrium mixture containing more than 95 per cent dihydroxyacetone phosphate. This result is due to the inability of isomerase to transform glyceraldehyde phosphate which is chemically combined with hydrazine or sulfite. In the absence of an interceptor, *e.g.* hydrazine, the isomerase activity of crude muscle extracts is far greater than the aldolase activity, as shown by the fact that synthetic glyceraldehyde phosphate (Fischer ester) is brought nearly completely into the equilibrium ester of triose phosphates before the condensation to hexose diphosphate has started (2).

In 1940, Herbert, Gordon, Sabrahmanyam, and Green, (9) described the preparation of purified aldolase, free from isomerase. They reported that the greater part of aldolase is insoluble in 33 per cent ammonium sulfate (33 gm of ammonium sulfate per 100 cc of solution). In testing different ammonium sulfate fractions for isomerase, we found that only a small part of this enzyme is precipitated by 33 per cent or even 36.4 per cent ammonium sulfate, while 90 per cent or more of it remains in solution. The bulk of the enzyme is precipitated, however, when the ammonium sulfate concentration is increased to 43 to 44 per cent. When this precipitate is redissolved in 20 per cent ammonium sulfate, it is found to contain only traces of aldolase and phosphatase. These traces are destroyed by 15 to 20 minutes heating at 55°, and the solution, freed from the bulky precipitate, contains a highly concentrated isomerase, relatively stable.

*Aldolase, Isomerase, and Myogen A*—While we were engaged in experiments on the separation of aldolase and isomerase, a paper of Engelhardt (10) appeared, claiming that aldolase could be obtained in a pure state by making use of the crystallization of the material called myogen A by Baranowski (11). Since this protein fraction forms at least 10 per cent of the total soluble muscle protein, this supposition taken together with Engelhardt's view that myosin is identical with adenylypyrophosphatase would dispose of more than half of the total muscle protein for only two enzymes, and confine all other enzymes to less than a half.

Actually crystals of myogen A made according to Baranowski contain

large amounts of aldolase as well as of isomerase. If the crystallization is repeated several times, with concentrations of ammonium sulfate not greater than 0.4 saturated, and the crystals washed with ice water, isomerase can be washed out. After five such recrystallizations crystals are obtained free from isomerase, containing aldolase in an amount of 0.70 unit per mg. Since Baranowski has reported that about 10 per cent of the protein of the original muscle extract can be obtained as crystallized myogen A, the figure of 0.7 unit per mg of protein is in good agreement with the 0.059 unit per mg of protein, shown initially by the muscle extract from which the myogen was prepared.

A great part of the aldolase present in the original extract is therefore still present in the enriched crystals. Nevertheless, the crystals are not

TABLE I  
*Aldolase, Isomerase, and Myogen A*

Protocol No	No of crystallizations carried out	Activities of solutions tested, units per mg protein					
		Original extracts and solutions made up from crystals			Mother liquors		
		Aldolase	Isomerase	Ratio	Aldolase	Isomerase	Ratio
IV	Before crystallization	0.02-0.06	Present				
	1	0.10-0.21	"				
	2	0.21					
	3	0.33	Present				
	5	0.46	0.31	0.70			
IX	Before crystallization	0.059	10.0	170			
	1	0.10	4.0	40	0.10	12.0	120
	2	0.33	6.5	20	0.024	7.5	310
	5	0.4-0.5	0	0	0.033	2.5	75
	6	0.70	0	0	0.15		

pure aldolase, since Warburg and Christian (12) recently obtained the crystallized ammonium salt of aldolase, exhibiting an activity of 4.35 units per mg, 6 times as high.

The data obtained in making the series of recrystallizations leading to crystals free from isomerase are shown in Table I. Aldolase was determined as in the preceding paper (4), triose phosphate formed from hexose diphosphate was trapped by KCN as the cyanhydrin, and with the purer preparations the curve of the equilibrium reaction was also determined. The initial velocity in presence and absence of KCN proved to be identical.

The appearance of the crystals after the second recrystallization, when they become colorless, remains the same in spite of the change in enzymatic activity. If it is borne in mind that according to Gralén (13) crystallized myogen A behaves as a homogeneous protein in the Svedberg ultracentri-

fuge, the findings that only the sixth part of it is aldolase stress the view that neither crystallization nor homogeneity of particle size is a sign of purity and uniformity of protein<sup>1</sup>

Engelhardt states in the paper already quoted (10) that he obtained, after four recrystallizations, a turnover number for aldolase of 1000. This figure, calculated in the same way as ours, corresponds to 0.30 unit per mg. This is in close agreement with the value obtained here after two and three recrystallizations, but this value is steadily increasing, and the 0.7 unit equal to a turnover number of 2200 may still go up somewhat in further recrystallizations.

*Separation of Triose Phosphate Isomerase from Hexose Phosphate Isomerase*—In testing the different fractions of muscle extract for activity of the two enzymes, it becomes immediately clear that the two enzymes are quite different. Hexose phosphate isomerase goes into the aldolase fraction, i.e. it is precipitated by 33 per cent ammonium sulfate, while our isomerase remains in solution. A purified isomerase preparation (step (2) below), which in a dilution of 1:20,000, equal to 1.8  $\gamma$  of protein, isomerizes 0.350 mg of P of triose phosphate in 4 minutes at 37°, has in a dilution of 1:40 (= 900  $\gamma$ ) no effect on hexose monophosphate. On the other hand, the aldolase fraction of the same preparation in a 1:7000 dilution transforms about one-fifth of 0.160 mg of fructose monophosphate into glucose monophosphate, in 4 minutes, while in a 1:700 dilution the equilibrium distribution for this amount of hexose monophosphate is nearly attained in 4 minutes. Less than 50  $\gamma$  of acetone powder of muscle extract transforms half of this amount of hexose monophosphate in 4 minutes. The claim of Iri is therefore completely unwarranted.

*Kinetics of Isomerase Reaction*—In Fig. 1 two experiments made at 20° are reproduced. The reaction was measured by rotation. At 38° the spontaneous decomposition of triose phosphate in times longer than 4 minutes interferes with an exact evaluation of the results. At 20° this decomposition is negligible during an hour, but the decrease in activity of the enzyme in this period is very clearly visible. The dash line is theoretical for a monomolecular equilibrium reaction, assuming the equilibrium constant  $K = (\text{dihydroxyacetone phosphate})/(\text{glyceraldehyde phosphate})$  to be 24. This curve is calculated from the equation

$$\text{Log}_{10} \frac{S - x_1}{S - x_2} = k(t_2 - t_1)$$

where  $S$  is that triose phosphate which *can* be transformed into dihydroxyacetone phosphate (96 per cent of the total), while  $x_1$  and  $x_2$  are the amounts

<sup>1</sup> For these experiments we had the assistance of Dr. Renate Junowicz-Kocholaty, to whom we wish to express our sincere appreciation.

transformed in the times  $t_1$  and  $t_2$  and  $k$  is the velocity constant. For derivation of the equation, see Meyerhof (14). Since the preparation contains at the beginning of the experiment 25 per cent dihydroxyacetone phosphate in the triose phosphate, equal to  $3.31 \times 10^{-3} M$  concentration in the enzymatic mixture, this value represents  $x_0$  at  $t_0$ .

From Fig. 1 it may be seen that only the first two or three points, up to about two-thirds of the total turnover, lie on the theoretical curve. All

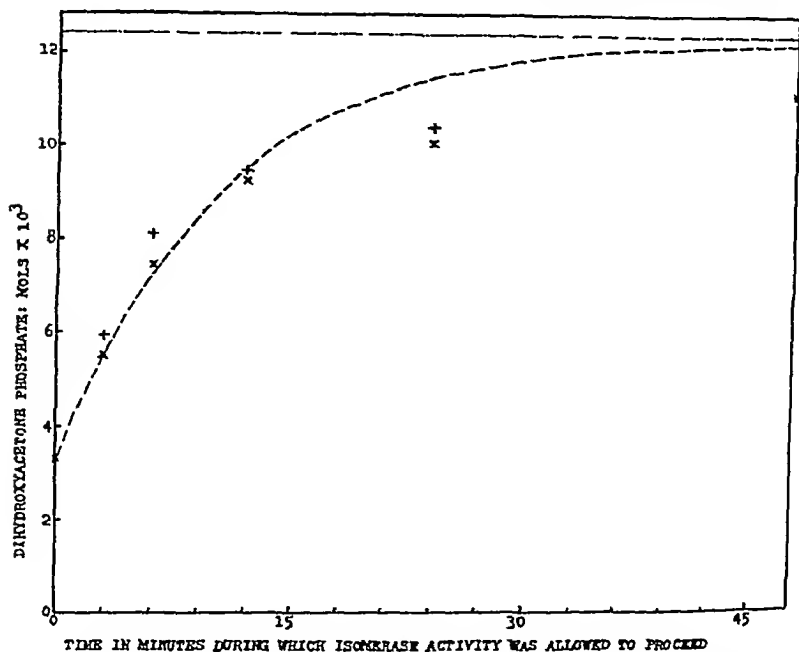


FIG. 1 Kinetics of the isomerase reaction. The dash line represents the theoretical curve for a monomolecular reaction, the dot and dash line, the equilibrium concentration of dihydroxyacetone phosphate. + and X are the experimental points of two experiments.

following points are much below. The same happens at  $38^\circ$ , if due allowance is made for spontaneous breakdown of triose phosphate.

On the other hand with both time and original concentration of glyceraldehyde phosphate constant, the greater the enzyme concentration the smaller is the initial activity *per mg* of protein, as calculated from the monomolecular equation. For example, with 1.063  $\gamma$ , 0.354  $\gamma$ , and 0.106  $\gamma$  of protein, 77, 57, and 22 per cent respectively of the original glycer-

aldehyde phosphate were transformed in 3 minutes. This corresponds to calculated initial activities of 36, 59, and 57 isomerase units respectively. In all but one of eight other experiments involving three enzyme dilutions, when the amount of enzyme was sufficient to bring about over 65 per cent transformation of the original glyceraldehyde phosphate in 3 minutes, the calculated initial activities were considerably lower than the initial activities secured in the simultaneous tests with less enzyme. Whether enzyme concentration is kept constant and time varied, or time kept constant and enzyme concentration varied, the reaction slows down more as equilibrium is approached than would be expected from the monomolecular equation.

As a corollary of these results, it should be noted that attainment of the final equilibrium distribution of the triose phosphate in a short period of time requires the use of 10 to 100 times as high enzyme concentrations as would be expected from the high activity values secured when less than 50 per cent of the original glyceraldehyde phosphate has been transformed within the test period.

The effect spoken of above is not due to a decrease in the intrinsic activity of the enzyme with increase in its concentration. If the initial glyceraldehyde phosphate concentration is also increased, in direct proportion to the increase in enzyme concentration, the percentage of original glyceraldehyde phosphate transformed in 3 minutes is held approximately constant, and the calculated initial enzyme activity is, if anything, greater for high than for very low enzyme concentrations.

The bearing of the velocity curve upon the calculation of the enzyme activity should not be overlooked. Since the curve starts with about 30 per cent dihydroxyacetone phosphate, and the turnover is measured approximately for the interval between 30 and 60 per cent dihydroxyacetone phosphate, the mean speed expressed in mg of P per minute is about one-half of that which would be expected for 100 per cent glyceraldehyde phosphate in the very beginning of the reaction. The turnover number therefore should be roughly doubled if it is to be compared with those of other enzymes in which the reaction starts with the pure initial product, or the end-product is continually intercepted or removed. The accuracy of the single determinations is not high enough to warrant treating all our data according to the formula, therefore we apply this correction only for the final figure. Moreover, certain triose phosphate preparations gave appreciably lower activity values than those obtained simultaneously with certain other preparations. It is thought that preparations giving low values may contain a condensation product which inhibits the reaction. The disintegration products of triose phosphate, methylglyoxal and inorganic phosphate, have no effect on isomerase activity.



*Purification of Isomerase*—(1) To each 100 cc of crude extract (1 part of rabbit muscle and 1.5 parts of glass-distilled water) were added 45 gm of solid ammonium sulfate, giving a 36.4 per cent ammonium sulfate solution (36.4 gm per 100 cc), or 0.67 saturated. The bulky precipitate was removed by rapid centrifugation, or by repeated filtration through qualitative paper in a cold room. 10 gm of solid ammonium sulfate were added to each 100 cc of the filtrate, giving a final ammonium sulfate concentration of 43.8 per cent or 0.81 saturated. The precipitate, which contained most of the enzyme, was collected by rapid centrifuging, or much more easily on a sheet of No. 50 Whatman paper by filtration through a Buchner filter set up in a cold room. *Care must be taken that this precipitate does not have a chance to dry.* The precipitate was redissolved, together with the fluid which could not easily be separated from it, in the amount of water required to bring the final ammonium sulfate to 20 per cent, and cleared by filtration through qualitative filter paper or glass wool. If the ammonium sulfate concentration was less than 20 per cent, sufficient amounts of a saturated solution of ammonium sulfate were added to bring the concentration to this value. The solution was heated at 55° for 10 or 15 minutes, and the bulky precipitate discarded. Frequently the heating was repeated for a second 10 minute period. The clear solution was stored in the cold. Values obtained for isomerase preparations secured in the above manner are shown in Table II.

(2) Addition of solid ammonium sulfate to preparations of the heated enzyme, secured as described in step (1), led to considerable denaturation, without increase in activity. However, the activity could be increased by addition of sufficient saturated ammonium sulfate to the heated enzyme to give a 0.677 saturated solution, the precipitate obtained by centrifuging at 5000 R.P.M. was discarded, saturated ammonium sulfate added to a final concentration of 43 per cent (0.80 saturated), and the second precipitate, which contains most of the isomerase, was centrifuged down. In some cases an additional precipitate was obtained by increasing the ammonium sulfate concentration to 46 per cent (0.86 saturated). The precipitates must be quickly redissolved in water, since otherwise they become increasingly denatured.

The results secured in some of these procedures are indicated in Table III.

Short dialysis of the heated enzyme and its ammonium sulfate fractions against distilled water down to 1 per cent ammonium sulfate may cause some loss of protein, although the activity per unit weight of protein is not greatly changed. Further removal of ammonium sulfate is deleterious to the enzyme.

*Electrophoresis Experiment*—A preparation secured by two successive

TABLE II

*Enzyme Activities of Preparations from Step (1)*

Protocol No	Heat treatment at 53	Age of enzyme at time tested	Enzyme protein used	P transformed per min †	Units per mg protein	
					Single values	Mean values
		<i>days</i>	<i>γ</i>	<i>γ</i>		
VI-II	1 × 10 min	1	0 86 (D)	40 0	47	49
	Same enzyme	2	0 86 "	41 5	48	
	"	9	0 65 "	34 3	53	
M-IV	1 × 10 min	1	1 72 "	81 3	47	63
M-II	2 × 10 "	1	0 78 "	61 3	78	
	Same enzyme	3	0 77 "	41 0	53	
	"	3	0 385 "	22 5	59	66
M-IV	2 × 10 min	1	1 25 "	71 3	57	
	Same enzyme	5	1 25 "	95 0		
B-VIII	2 × 10 min	7	0 048 (N)	5 34	115	55
B-XI	1 × 15 "	8	0 096 "	6 32	66	
B-XIV	1 × 15 "	8	0 080 (B)	5 14	64	
	Same enzyme	8	0 080 "	3 85	48	
	"	8	0 080 "	4 26	53	

\*D signifies that the protein was estimated from dry weight, N from nitrogen × 6.25, and B from biuret determinations. Actual dry weight to nitrogen ratios ranged from about 7.5 to 9.

†Protocol numbers preceded by M indicate a 4 minute test period, and estimation of P transformed by the rotation method, protocol numbers preceded by B indicate a 3 minute test period and estimation of P transformed by the alkaline hydrolysis method.

TABLE III

*Enzyme Activities of Various Ammonium Sulfate Fractions of Heated Enzyme (Step (2))*

Protocol No	(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> fraction secured	Age of enzyme at time tested	Enzyme protein used*	P transformed per min	Units per mg protein	
					Single values	Mean values
		<i>days</i>	<i>γ</i>	<i>γ</i>		
M-IV	A (33-43%)	1	0 64 (D)	46 5	73	76
	" dialyzed to 1.6% (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	1	0 64 "	44 3	69	
	Same enzyme	5	0 58 "	50 5		
M-V	32-42%		1 12 "	64 5	58	91
	Same enzyme		0 56 "	33 0	59	
	42-46%		0 65 "	69 5	107	
	Same enzyme		1 00 "	74 5	75	
B-XI <sub>3</sub>	Over 39.3% (supernatant)	7	0 056 (N)	7 75	138	
	" 48.6% "	7	0 0184 "	2 56	139	
B-XI <sub>4</sub>	" 43% "	2	0 056 "	8 69	158	

\* See the foot-note to Table II

ammonium sulfate precipitations was subjected to electrophoretic measurement in phosphate at pH 6.6 in the Tiselius apparatus, after dialysis to 1.6 per cent ammonium sulfate (Preparation M-IV, Table III). It showed three fractions of 3 per cent (fast), 30 per cent (middle), and 60 per cent (slow). Since it was partly inactivated during the electrophoresis, and even the portion in the bottom of the tube, not exposed to the outside buffer, had lost 30 per cent of its original activity, it is uncertain whether one of these fractions represented the pure enzyme\*. Actually by the next step and occasionally by further ammonium sulfate precipitation,

TABLE IV

*Preparations Secured by Cu(OH)<sub>2</sub> Adsorption and Phosphate Buffer Elution (Step 5)*

Protocol No	Treatment of preparation	No of elution	Enzyme protein used*	P trans formed per min	Units per mg protein	
					Single values	Mean values
M	A, as in step (1), then dialyzed to 0.04% (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	None made	0.71	27.5	38	43
			0.36	16.8	47	
	B, 2 cc A + 4 cc Cu(OH) <sub>2</sub> , eluted with 4 cc buffer	1st	0.23	44.8	193	170
			0.09	13.1	147	
M	B, repeated 7 days later	1st	0.18	21.5	121	
			0.73	58.3	80	
	As for B except 3 cc buffer used for elution	1st				
B-IX	C, 1 cc A + 2.8 cc Cu(OH) <sub>2</sub> , eluted with 3 cc buffer	1st	0.25	23.6	94	
		2nd	0.15	20.8	137	
	5 cc dialyzed heated enzyme + 5 cc Cu(OH) <sub>2</sub> , eluted with 5 cc buffer	1st	0.094	6.35	68	163
		2nd	0.041	5.76	140	
			0.0082	1.53	186	
		3rd	0.165	7.80	120	
			0.013	1.53	118	
						119

\* The protein was estimated from nitrogen  $\times 6.25$

fractions could be obtained which exhibited about twice as great activity as that shown by the preparation subjected to electrophoresis, although never in stable form. It is probable, therefore, that less than half of this preparation represents the enzyme in the pure state.

(3) Attempts to purify the enzyme still further by adsorption on kaolin, Al(OH)<sub>3</sub>, etc., proved unsuccessful, but adsorption on washed Cu(OH)<sub>2</sub>, as used by Herriott (15) for purification of pepsinogen was very effective. The enzyme adsorbed on Cu(OH)<sub>2</sub> is eluted by phosphate buffer at pH 7.2.

\* The measurement was made in the Electrophoresis Laboratory of Dr. D. H. Moore, College of Physicians and Surgeons, Columbia University, to whom we give our best thanks.

These elutions when tested immediately show the highest activity so far obtained and are completely water-clear. They keep a little better if some ammonium sulfate is added, but even in the presence of ammonium sulfate they deteriorate after some days in the cold room (Table IV).

Although owing to instability it cannot be stated that we obtained the pure enzyme in this way, this step may lead to the final purification if more thoroughly investigated. The activity obtained so far of a turnover of about 500,000 is not only the highest which we secured with this enzyme, but also one of the highest exhibited by any enzyme. In terms of initial velocity, with 100 per cent glyceraldehyde phosphate as substrate, this would correspond to a turnover number of about 1,000,000.

*Yield*—The total yield of isomerase from muscle and the degree of purity of the initial steps are somewhat increased by shaking the ground up tissue with 4 or 9 volumes of water instead of 15 volumes. The units obtained per gm of muscle were 125 with 15 volumes of water, 187 with 4 volumes, 198 with 9 volumes, and 268 with the same volume after semi-homogenizing the muscle.

The units per mg of protein increase in the same order from 2.2 to 6.6. The recovery of isomerase from muscle extract up to the end of step (1) is about 40 to 60 per cent, while the following steps give different results, depending upon the details of the procedure. The elution from  $\text{Cu}(\text{OH})_2$  can be carried out with little loss. Generally 25 to 40 per cent of the protein contained in the dialyzed solution was extracted by two consecutive elutions, while the activity rose 2 to 3 times.

#### DISCUSSION

If we assume that we have succeeded in bringing isomerase to a nearly pure state, the turnover number of the pure enzyme for an assumed molecular weight of 100,000 would be a little over 1,000,000. This would indicate that about 4 per cent of the protein of muscle extract would be isomerase. From the figures of Warburg and Christian for crystallized aldolase it may be calculated that 15 per cent of the protein of muscle extract is aldolase.

Without isomerase only half of the hexose diphosphate would be oxidized by way of glyceraldehyde phosphate to pyruvic acid, while the other half would be reduced by way of dihydroxyacetone phosphate to  $\alpha$ -glycerophosphate. This dismutation, which plays a major rôle only in the induction period of fermentation, is rather slow. The oxidation-reduction of the stationary state between glyceraldehyde phosphate and acetaldehyde or pyruvic acid can only function with the help of isomerase. Nevertheless, it is interesting that the equilibrium point of triose phosphate lies so far to the side of the ketotriose, although it forms a metabolic blind alley, while the aldotriose is the thoroughfare of the pathway of carbohydrate

breakdown The large amounts of isomerase present in muscle and the extremely rapid turnover number exhibited by this enzyme may be thought of as mechanisms developed by the muscle cells to deal with the metabolically unfavorable triose phosphate equilibrium

#### SUMMARY

The enzyme triose phosphate isomerase can be easily separated from aldolase and hexose phosphate isomerase

The crystallized myogen A of Baranowski contains aldolase as well as isomerase The latter can be removed by repeated recrystallizations, but even after six recrystallizations only a very small part of the crystalline protein is aldolase

Purified isomerase can be obtained from the mother liquors of aldolase by fractionation with ammonium sulfate By adsorption on  $\text{Cu}(\text{OH})_2$  and elution one obtains an enzyme having a "turnover number" at  $38^\circ$  of about 1,000,000 Owing to the instability of this enzyme preparation it is uncertain whether this activity corresponds to the pure enzyme

The kinetics of the isomerization exhibit a slower rate as equilibrium is approached than that calculated from the law of mass action The reason for this apparent inhibition remains unexplained

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# DETERMINATION OF CITRULLINE AND ALLANTOIN AND DEMONSTRATION OF CITRULLINE IN BLOOD PLASMA

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## I CITRULLINE

Fearon (1) introduced the use of diacetylmonoxime,  $\text{CH}_3\text{C}(\text{NOH})\text{COCH}_3$ , in strongly acid solution (trichloroacetic) as a reagent for the colorimetric determination of citrulline. The color produced is a reddish yellow peach shade. Fearon recognized that allantoin and urea, when present, vitiated the results by producing colors which are somewhat more yellowish.

Gornall and Hunter (2) used Fearon's reaction to measure citrulline after removal of urea with urease. They realized that the urease contributed a rather appreciable blank. We have found that allantoin or allantoic acid and canavanine present in commercial preparations of urease are responsible for most, if not all, of this blank and have removed these materials by dialysis (3). When dialyzed urease is used to destroy urea, no urease blank need be run.

Interference by allantoin is prevented by adsorbing the citrulline with Amberlite at pH 6 to 7. Allantoin is not adsorbed. The difference in chromogenic material before and after adsorption is a measure of the citrulline. Interference by other plasma extractives besides allantoin is also eliminated by this procedure.

It is desirable to keep at a minimum the color formed by allantoin in the mixture analyzed before adsorption of the citrulline. This object has been attained by forming the color in a solution acidified with a 1:3 mixture of sulfuric and phosphoric acids, and heating for 10 minutes. Under these conditions color development from citrulline is almost maximal, while the amount of color formed by allantoin is only about one-fiftieth as much as that from equimolar amounts of citrulline.

The nature of the carbamido-diacetyl reaction and the structure of the products formed remain unknown. Formation of the colored product is apparently favored by the presence of an oxidizing agent and to some extent by light. The product, however, is at the same time photolabile and is destroyed by excess oxidant. Color formation takes place slowly at room temperature.

The following observations on the nature of the alternative reagents and the subsequent discussion of the specificity of the reaction relate to

the mechanism of the reaction only in so far as they characterize the structure of the reactants

Diacetyl,  $\text{CH}_3 \text{ CO CO CH}_3$ , or diacetyldioxime,  $\text{CH}_3 \text{ C NOH C NOH CH}_3$  (dimethylglyoxime), and diacetylmonoxime, each in equimolecular concentrations, when heated in acid solution with a given carbamido compound give colors with absorption curves of the same shape and with maxima at the same wave-length. However, the optical density is slightly greater when diacetylmonoxime is used. Benzoylacetyl,  $\text{C}_6\text{H}_5 \text{ CO CO CH}_3$ , and its monoxime,  $\alpha$ -isonitrosopropiophenone,  $\text{C}_6\text{H}_5 \text{ CO C NOH CH}_3$ , also react with carbamido compounds, and the use of  $\alpha$ -nitrosopropiophenone has proved of value in the determination of urea, as will be shown in a subsequent publication. It offers, however, no advantage for the determination of citrulline.<sup>1</sup>

Lang observed (4) that the diacetyl color reaction with guanidine derivatives in alkaline solution takes place only when an alkyl group is present on at least one end of the diketone group. We have found the same to be true of the diacetyl color reaction with carbamido derivatives in acid solution. Neither benzil,  $\text{C}_6\text{H}_5 \text{ CO CO C}_6\text{H}_5$ , nor *p*-benzoquinone (which may be considered to be a vinylogue of diacetyl) gives color when heated in acid with citrulline, urea, or allantoin.

A number of the commercially available zeolites and cation exchange resins adsorb substances from solution more or less selectively. The specificity of the adsorption depends largely on the basicity of the substances presented for adsorption and the affinity and to some extent the capacity of the adsorbent for basic groups. Of those substances which give a strong carbamido-diacetyl reaction, citrulline is the most basic one likely to be present in biological material. (Stronger bases such as arginine would be adsorbed but do not give the carbamido diacetyl reaction.) The following procedures for the determination of citrulline and allantoin utilize these cation exchange materials and thereby increase the specificity of the methods. Conditions for pretreatment of adsorbents have been worked out which permit adsorption of citrulline but not of the less basic substances (such as allantoin).

When urease is used to remove urea, it is advantageous to have the enzyme act in the presence of KCN. Commercial preparations of urease contain a number of other enzymes, some of which alter the specificity of the method unless their action is inhibited by KCN. Although the use of crude crystalline urease (5) might largely overcome the need for using KCN, the convenience and economy of dissolving the dialyzed urease in cyanide solution have led us to adopt this alternative. Dried

<sup>1</sup> The color produced by reaction of citrulline with benzoylacetyl or its monoxime is a gray-purple.

dialyzed urease (3) has been stored in an ice box for 2 years without loss of more than 10 per cent of its activity

### *Method for Citrulline*

#### *Apparatus—*

*Dialysis units* as described by Hamilton and Archibald (6)

*Adsorption columns* Columns similar to the one illustrated in Fig 1 are prepared Amberlite IR-100 (analytical grade<sup>2</sup>) fills the bottom 2 inches of the column and the rate of filtration should not be faster than 3 drops per 2 seconds Before use, each column is flushed in succession

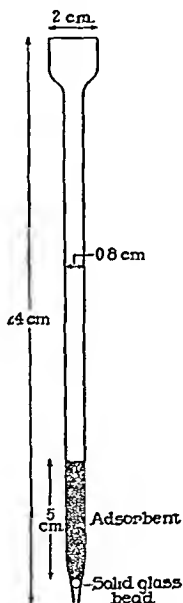


FIG 1 Adsorption column for removal of citrulline

with 10 cc of 10 per cent NaCl solution, 5 cc of  $H_2O$ , 10 cc of 12 N HCl, and 25 cc of  $H_2O$ . It is then dried with 5 cc of alcohol followed by 5 cc of ether and a current of air. Acetone may be used in place of alcohol and ether. The columns may be used at least ten times before being recharged with fresh adsorbent if they are flushed out before each use with salt, acid, water, alcohol, ether, and air.

<sup>2</sup> Amberlite was obtained from the Resinous Products and Chemical Company, 225 West Washington Square, Philadelphia



Zeo-Karb H<sup>3</sup> may be used in place of Amberlite, and for some purposes may be preferred. In this case the newly charged columns are washed with 12 N HCl until the washings are no longer yellow and allowed to stand for 3 hours or overnight in contact with the HCl before being washed again with salt, acid, water, alcohol, and ether. This treatment with concentrated acid alters somewhat the properties of the adsorbent but renders it better suited to the needs of this analysis.

#### Reagents—

*Sulfuric-phosphoric acid mixture*, 1 volume of concentrated sulfuric acid and 3 volumes of syrupy phosphoric acid

*Diacetylmonoxime*, 3 per cent solution in water

0.02 N H<sub>2</sub>SO<sub>4</sub> (approximate), 10 cc of concentrated H<sub>2</sub>SO<sub>4</sub> diluted to 1800 cc

0.4 M KCN, pH 7.2. Dissolve 1 gm of KCN in 18 cc of H<sub>2</sub>O. Add cautiously in a hood, with stirring, 20 cc of M NaH<sub>2</sub>PO<sub>4</sub>. Store in an ice box.

*Dialyzed urease*<sup>4</sup> prepared according to the directions of Archibald and Hamilton (3), 2 per cent solution in 0.4 M KCN buffered with NaH<sub>2</sub>PO<sub>4</sub> to pH 7.2

*Stock standard* 10.00 mg of citrulline per 100 cc

*Working standard* 0.01 mg per cc, 1 cc of stock standard is diluted to 10 cc with water

#### Procedure

0.56 cc of the 2 per cent urease is added to 4 cc of plasma and the mixture allowed to stand at room temperature for 20 minutes to hydrolyze urea. (Plasma from uremic patients is allowed to incubate 1 hour.) 4 cc of the plasma thus digested are then dialyzed for 2 hours against 10 cc of 0.02 N H<sub>2</sub>SO<sub>4</sub> in the apparatus described by Hamilton and Archibald (6)<sup>5</sup>. 4 cc of the dialysate, 2 cc of the sulfuric-phosphoric acid mixture, and 0.25 cc of diacetylmonoxime reagent are pipetted into test-tubes and mixed. The remaining dialysate is adjusted with the help of indicator paper to a pH between 6.0 and 7.0 by addition of 18 N NaOH.

<sup>3</sup> Zeo-Karb H is obtained from The Permutit Company, 330 West 42nd Street, New York

<sup>4</sup> Squibb's double strength urease was employed for the preparation of the dialyzed urease

<sup>5</sup> Dilute sulfuric acid instead of water is used for dialysis in order to stop the action of enzymes present in the jack bean urease preparation before appreciable amounts of allantoin or uric acid have been converted to allantoinic acid or to other compounds which give a positive carbamido-diacetyl reaction. When 4.0 cc of the 4.56 cc of urease digest are dialyzed against 10 cc, the protein free solution, at equilibrium is exactly a 1:4 dilution of the plasma concentration.

solution, and passed through an adsorption column to remove citrulline. 4 cc of the filtrate are mixed with monoxime reagent and acid in the same manner as is the untreated dialysate. Standards and reagent blanks are prepared with 4, 2, 1, 0.5, or 0 cc of standard solution and 0, 2, 3, 3.5, or 4.0 cc respectively of water, and the same amount of acid and monoxime as was used for the samples. After mixing the solution, the tubes are capped with glass marbles or bulbs and heated in a boiling water bath, from which light is excluded. After exactly 10 minutes, they are set in a covered container to cool in water. Once the tubes are heated, it is essential that they be protected from light until readings are made. Approximately 10 minutes after the end of the heating period, the optical densities are read in a photometer with light of wave-length of 490 m $\mu$ . The photometer scale is set at zero optical density with the reagent blank.

*Calculation for Citrulline*—The optical density of each standard is plotted against the mg of citrulline, and the citrulline equivalents of the samples are read from this curve. The citrulline equivalent of the Amberlite or Zeo-Karb filtrate is subtracted from the citrulline equivalent of the untreated dialysate to give the true weight of citrulline. Since 4 cc of dialysate are equivalent to 100 cc of plasma, the mg of citrulline found in 4 cc of the dialysate is multiplied by 100 to obtain the concentration in mg per 100 cc of plasma.

$$\text{Mg citrulline per 100 cc plasma} = 100 (D - F)$$

$D$  = citrulline equivalent (in mg) of color obtained on heating 4 cc of dialysate of urease-treated plasma

$F$  = citrulline equivalent (in mg) of color obtained on heating 4 cc of dialysate which has been treated with Amberlite or Zeo-Karb

### *Discussion of Method*

As pointed out by Gornall and Hunter (2), to obtain consistent results it is necessary to adhere rigidly to a given set of conditions. They noted in their method, as we did in ours, that a calibration curve is necessary, because optical density is proportional to concentration only over a narrow range and there is relatively too little color with the lower concentrations. If the solutions (after being heated) are exposed for a few minutes to light of ordinary laboratory intensity, appreciable fading of color results. The per cent of color lost in a given interval is greater the lower the concentration of citrulline. This light sensitivity is decreased by the presence of some unrecognized constituent (not glycine, arginine, ammonium carbonate, or glucose) of plasma. Hence unless tubes are protected from light, color development appears to obey Beer's law better when it takes place in plasma dialysates than when solutions of pure citrulline are used. For some purposes it has proved convenient to construct calibra-

tion curves by use of solutions of stock citrulline diluted with Amberlite or Zeo-Karb filtrates of dialysates of urease-treated plasma. If, however, the heated standards are protected from light, dilution of citrulline with distilled water has proved adequate.

The advantages of this method over those previously published are as follows. A high yield of a concentrated protein-free filtrate of plasma or its equivalent is available by use of the dialysis technique. By use of dialyzed urease, the high blanks obtained with commercial preparations are avoided. By use of a high concentration of KCN during urease action, formation of chromogenic by-products from allantoin and uric acid is avoided. By use of the high concentration of phosphoric acid the color formed by action of the reagent on allantoin in 10 minutes is minimized. This same acid mixture is more satisfactory for development of color with citrulline than is either acid alone. Except for Abelin (7), who described a method for the rapid approximate determination of serum urea, previous investigators, using the carbamido-diacetyl reaction, have employed persulfate to achieve maximum color intensity. The use of this oxidant is attended subsequently by a rapid fading of color. When the heating takes place in a mixture of sulfuric and phosphoric acids, addition of persulfate becomes unnecessary and the color obtained fades much less rapidly than when persulfate is used.

Adsorption of the chromogen on Amberlite is an addition which increases enormously the specificity of the determination.

The method has been applied successfully to the determination of citrulline in dialysates of enzymatic hydrolysates of protein.

Attempts were made to simplify the determination of citrulline by passing plasma through Zeo-Karb, washing the columns with 0.3 per cent saline, then with water, then measuring citrulline in an acid eluate of the Zeo-Karb. However, under conditions which were adequate to achieve quantitative adsorption of citrulline from plasma, elution was incomplete.\*

#### *Specificity of Citrulline Method*

Fearon (1) and Gornall and Hunter (2) have considered in detail the specificity of the color formation with diacetylmonoxime in acid. It should be noted that to interfere with the specificity of the present citrulline method a substance after treatment with urease in KCN must (a) dialyze through cellophane, (b) cause appreciable absorption of light of wave-length 490 m $\mu$  after being heated for 10 minutes under the con-

\* A small fraction of the urea present is adsorbed on Zeo-Karb H and is eluted with the citrulline by acid. The peach color of citrulline was therefore partly masked by the yellow from the urea. Nevertheless the absorption curve indicated that the citrulline like compound was present in the eluate.

ditions specified for the analysis, and (c) be partly or completely adsorbed by Amberlite or Zeo-Karb. The peach color formed when *citrulline* is present is visibly different from the more yellow color formed with a number of other substances which give a positive carbamido-diacetyl reaction. The product formed with *citrulline* has an absorption maximum at wave-length 490  $m\mu$ . *Citrulline* is not attacked by urease and is completely adsorbed by the Amberlite or Zeo-Karb, these properties serve to separate *citrulline* from other plasma constituents that give color with diacetylmonoxime.

*Urea* is removed completely from normal plasma by 20 minutes incubation with the specified amount of urease. The ammonium carbonate formed from blood urea does not interfere with either the adsorption of *citrulline* or the diacetyl color reaction. The large amount of ammonia formed on incubating buffered urine with urease must be removed prior to adsorption of *citrulline* or the large excess of ammonium ion would interfere with the adsorption of the less basic *citrulline*. The removal of ammonia may be accomplished either by vacuum distillation at pH (8) or by passing the solution through a column of Decalso (Folin permutit) arranged as illustrated in Fig. 1. The Decalso does not adsorb *citrulline* in the presence of as much salt as is present in plasma dialysates and the amount used in the column for removal of ammonia removes insignificant amounts of chromogen from dialysates, and may be freed from ammonia for repeated use by treating it with 10 cc. of 10 per cent solution of NaCl followed by water.<sup>7</sup>

*Allantoin*,  $\text{NH}_2 \text{CO NH} \text{CH CO NH CO NH}$ , like urea, yields a product having a yellow color with an absorption maximum at 480  $m\mu$ . However, because of the high concentration of phosphoric acid in the acid mixture used, the amount of color formed during 10 minutes heating is inappreciable unless relatively large quantities of allantoin are present. Further, allantoin is not adsorbed by Amberlite and only 5 to 8 per cent of that present is adsorbed by Zeo-Karb.

In the absence of KCN, Squibb's preparation of urease, even when partially purified by dialysis, forms from allantoin a substance yielding with diacetylmonoxime a product with an absorption curve indistinguishable from that obtained with *citrulline*. In the presence of 0.04 M KCN no chromogen was formed by the action of the urease preparation. Allantoin, therefore, does not interfere with the specificity of the *citrulline* determination.

<sup>7</sup> As shown by Dubnoff (9), 3 per cent NaCl will elute arginine from Decalso. Ammonia, however, is but slightly eluted by 3 per cent NaCl, although almost completely by 10 per cent solution.

*Uric acid* does not form a colored product under the conditions of the analysis. In the absence of KCN the dialyzed preparation of urease used in the present analysis acting on uric acid forms a product which, on being heated with diacetylmonoxime, yields a colored product with an absorption curve like that given by urea or allantoic acid. In the presence of 0.04 M KCN, however, uric acid, like allantoin, yields no chromogens on treatment with urease. In this connection it may be noted that uricase and allantoinase have been said to occur in soy bean preparations (10, 11). Ling (12), however, says that soy bean contains no allantoinase. There appear to be no data in the literature indicating whether these enzymes accompany the urease preparation made from jack beans.

*Allantoic acid*,  $(\text{NH}_2 \text{ CO NH})_2 \text{CH COOH}$ , does not appear to have been considered by previous workers who investigated the specificity of the carbamido-diacetyl reaction. Allantoic acid was prepared according to the directions of Young and Conway (13). On being heated for 10 minutes in the presence of phosphoric acid, it gives (unlike allantoin) appreciable color with diacetylmonoxime. The absorption curve of the product is similar to that obtained with urea, or with allantoin after prolonged heating. Allantoic acid is not adsorbed by Amberlite or Zeo-Karb, and hence does not interfere in the present citrulline analysis.

*Alloxanic acid*,  $\text{NH}_2 \text{ CO NH CO CO COOH}$ , the first product of action of alkali on alloxan, also yields a positive carbamido diacetyl reaction and a color similar to that given by allantoic acid. Alloxanic acid is not adsorbed by Zeo-Karb or by Amberlite.

*Alloxan*,  $\text{CO CO CO NH CO NH}$ , heated under the conditions outlined for citrulline determination, yields a product with a golden yellow color, the shade of which is indistinguishable from that of the products from allantoic acid or urea. However, the intensity of the color is only 1/200 that of an equal weight of citrulline and the chromogen is not adsorbed by Zeo-Karb or Amberlite.

Alloxan reacts with KCN slowly at pH 6, rapidly at higher pH to yield a product which reacts with diacetyl yielding 70 times as much color in 10 minutes heating as is given by the alloxan from which it is derived. The chromogenic product is not urea. The maximal amount of the product formed by action of KCN on a given weight of alloxan yields with diacetyl one-third the amount of color given by an equal weight of citrulline. The absorption curve given by the product is similar to that given by urea and allantoic acid. This reaction with KCN is accompanied by liberation of 0.5 mole of  $\text{CO}_2$ . In a concentration of 0.05 M KCN at pH 7 the reaction is complete within 2 minutes. The structure of the product is unknown. These findings are the bases of two of four methods for the determination of alloxan to be published shortly.

When treated with urease, alloxan forms a chromogen which yields a faint color that has an absorption curve like that given by citrulline, with its maximum at  $490\text{ m}\mu$ . The reaction proceeds slowly at room temperature and is not attended by a liberation of  $\text{CO}_2$ . Neither alloxan nor the chromogen formed on treatment with urease or KCN is adsorbed by Zeo-Karb.

*Alloxantin*,  $(\text{CO NH CO NH CO C OH})_2$ , is not adsorbed by Amberlite. Heating  $1.652\text{ mg}$  of it under the specified conditions for 10 minutes gives an optical density of  $0.164$  at wave-length  $480\text{ m}\mu$ . The color produced is a yellow like that given with urea.

*Parabanic acid*,  $\text{NH CO NH CO CO}$ , is not adsorbed by Amberlite. Heating  $1.384\text{ mg}$  under the specified conditions for 10 minutes gives an optical density of  $0.940$  at the wave-length of the absorption maximum, viz  $480\text{ m}\mu$ .

As pointed out by Gornall and Hunter (2) both *methylurea* and *phenylurea* yield with the diacetyl reagent colors which are almost indistinguishable from that obtained with citrulline. These compounds are partly removed from solution by the adsorbents and are the only compounds known to interfere appreciably with the specificity of the citrulline test. Zeo-Karb, even after overnight treatment with  $12\text{ N HCl}$ , adsorbs 80 to 85 per cent of phenylurea from dilute aqueous solutions. Amberlite, on the other hand, adsorbs only 40 per cent under the same conditions. Amberlite also removes 50 per cent of the methylurea, whereas treated Zeo-Karb removes only 20 to 30 per cent.

As pointed out by Barker (14), *thymol* interferes and gives with diacetylmonoxime a color resembling somewhat that obtained with citrulline. Unlike citrulline, thymol is only partially adsorbed by Zeo-Karb. *Cresol* interferes less than does thymol and *phenol* is almost without effect on the reaction.

Traces of *protein* yield a pink color easily distinguished from that given by citrulline. It is a wise precaution to add a drop of 30 per cent sulfosalicylic acid to a  $1\text{ cc}$  aliquot of each dialysate to make sure that no protein has escaped from the dialysis cell.

*Buret*, both before and after treatment with urease, yields a positive carbamido-diacetyl reaction with a color similar to that given by urea.

*Thiourea* yields no color with the diacetyl reagent during 10 minutes heating and almost inappreciable color during a 60 minute heating period.

Neither *glutathione*, *ergothioneine* (thioneine), *glutamyltyrosine*, *glycylglycine*, *proline*, *histidine*, *carnosine*,<sup>\*</sup> *anserine*,<sup>\*</sup> *glutamine*, *hippuric*

\* Carnosine and anserine were made available through the kindness of Dr. D. W. Wilson, University of Pennsylvania.

*acid*, *p-aminohippuric acid*, *uric acid*, *benzimidazole*, nor *urethane* gives color on being heated in the prescribed acid mixture with diacetyl reagent. Neither *barbituric acid*, *phenobarbital*, *nembutal*, *caffeine*, nor *hydantoin*,  $\text{NH CO NH CH}_2 \text{ CO}$ , gives appreciable color under the 10 minute heat-

ing conditions outlined for citrulline. When the heating period is prolonged or when the 2 cc of phosphoric-sulfuric acid mixture are replaced by 18 N  $\text{H}_2\text{SO}_4$ , barbituric acid yields a light yellow color and hydantoin yields a color similar to that obtained with citrulline.

*Phenobarbital and nembutal* (sodium ethylmethylbutyl barbiturate) yield on 1 hour's heating in the phosphoric-sulfuric acid mixture only 3 per cent of the color given by an equal weight of barbituric acid.

*Presence of Citrulline in Plasma*—Gornall and Hunter (2) were aware of the fact that blood filtrates contained small amounts of chromogenic material which was different from urea and citrulline. Ormsby (15) noted that the color obtained on heating blood filtrates in acid with diacetylmonoxime was not exactly the same as if urea alone were present and he mentioned "citrulline or other material" as the cause. Barker (14) noted that Decalso (Folin permutit) removed from blood filtrates some of the chromogenic material which was not urea. He concluded, however, that the material removed was probably not citrulline. This conclusion was based on the shape of the absorption curve obtained with filtrates containing all of the urea. Had urea been removed, the presence of citrulline and (in the case of dog blood filtrates) allantoin would probably have been more apparent.

The conclusion that the chromogen measured in urease-treated plasmas as citrulline is indeed citrulline rests on the following evidence: (1) The color yielded by citrulline, urea and phenylurea or high concentrations of hydantoin in the diacetyl reaction is distinctive. The difference in color obtained with urea or allantoinic acid (golden yellow) on the one hand and citrulline (peach) on the other is more readily appreciated by the eye than by photoelectrometric readings. For the difference in color apparent to the eye the adsorption curves are remarkably close. (2) Of those few substances which yield a color similar to that obtained with citrulline, only citrulline is known to be removed completely by Amberlite or Zeo-Karb. Hydantoin, methylurea, and phenylurea are partly removed.

When fresh Zeo-Karb is pretreated with dilute sulfuric acid, instead of 12 N HCl as recommended in the above procedure, it removes all of the phenylurea and nearly all of the methylurea from solution, whereas Amberlite removes only 40 to 50 per cent of the phenyl- and methylurea. Since the amount of chromogen (10 minutes heating), left in the filtrate from urease-treated plasma dialysates is almost the same after treatment with Amberlite as it is after passage through sulfuric acid-treated Zeo

Karb (which removes all of the phenylurea and nearly all of the methylurea), one may conclude that not more than an insignificant portion of the material measured as citrulline can be either phenylurea or methylurea.

Although 5 to 8 per cent of the allantoin present is adsorbed when Zeo-Karb is used, the optical density attained in 10 minutes heating of the analyzed solution is not thereby appreciably reduced unless relatively enormous quantities of allantoin are present. The color given by urease-treated dialysates of most human plasmas is indistinguishable from that obtained with citrulline. Almost all of the chromogen in dialysates of human plasma and all of the chromogen yielding the characteristic color in dog plasma dialysates are removed by Amberlite or Zeo-Karb.

Determinations of citrulline content in aliquots of dog and human plasma started within 30 minutes after the blood was drawn gave the same results as determinations on other aliquots of the same plasma after storage in the ice box for 1 and 2 weeks. This would suggest that the citrulline found was not the result of proteolysis or autolysis of the plasma protein. Nevertheless all the plasma values recorded in Table I were obtained on plasma from freshly drawn blood.

The possibility that the citrulline found in plasma arises from plasma protein or any other constituent of plasma as a result of the action of proteolytic enzymes in the urease preparation has been eliminated by the following two observations. (1) Plasma which had not been treated with urease was passed through Zeo-Karb and the column was washed with 0.3 per cent NaCl solution, then with water. The chromogen was eluted with a mixture of 1 part of the phosphoric-sulfuric acid mixture and 2 parts of water. The eluted material gave the peach color characteristic of citrulline when heated in acid with diacetylmonoxime.<sup>6</sup> (2) Plasma was dialyzed against a large volume of water to remove preformed citrulline. The protein was then treated with urease in KCN for 1 hour and redialyzed. The amount of citrulline found in the second dialysate was unappreciable.

There is present, in the protein-free dialysates of some human and dog plasmas, a small amount of material which gives a color in the carbamido-diacetyl reaction that has an absorption maximum at a wave-length higher than that of citrulline (490 m $\mu$ ) and near that for plasma protein (500 to 510 m $\mu$ ). The dialysate of dog plasma represented in Fig. 2 is an example. The color may perhaps be due to a polypeptide or a di- or tripeptide. This component is partly adsorbed by Zeo-Karb and Amberlite. As a result some plasmas will yield an apparent citrulline value which is too high. The maximal positive error from this source is about 15 per cent, usually the error is negligible.

The fact that dialysates of urease-treated normal human plasma lose



by far the greater part of their chromogen (10 minutes heating) on passage through the adsorption columns indicates that (1) the total of the concentration of alloxanic acid, allantoic acid, biuret, methylurea, or phenylurea present in normal human plasma must be small, and (2) the concentration of KCN employed during the urease incubation is adequate to prevent formation of chromogen from allantoin and uric acid

## II DETERMINATION OF ALLANTOIN IN PRESENCE OF CITRULLINE

When both allantoin and citrulline are present, the amounts of each may be determined without using more of the sample than is needed to determine citrulline alone

### *Method*

The apparatus<sup>9</sup> and reagents are those described above for citrulline  
*Allantoin standard* A stock solution containing 0.10 mg per cc keeps at least a week if stored in the ice box

### *Procedure*

Standards containing allantoin instead of citrulline are set up as outlined above under the citrulline method. These are heated for 10 minutes, together with the citrulline standards, blank, and dialysates of samples (both with and without Amberlite treatment). Readings are taken at  $\lambda = 490 \text{ m}\mu$ , and the citrulline is calculated as outlined above.

The tubes containing the blank, the allantoin standards, and the portions of the dialysates treated with Amberlite are then heated a second time, this time for 50 minutes. After being cooled in the absence of light, the transmittance or optical density is read at  $470 \text{ m}\mu$ . The standard curve for allantoin is constructed by plotting the mg of allantoin in each known sample against the corresponding increase in optical density resulting from the additional 50 minutes heating. The allantoin equivalents of the samples after 60 minutes heating and after 10 minutes heating are read from the standard curve. The difference gives the figure for allantoin. The amount of allantoin found in 4 cc of filtrate is the amount present in 1 cc of plasma.

\* When allantoin determinations are to be made, the use of Amberlite as an adsorbent is preferable to the use of Zeo-Karb. Amberlite after being pretreated as specified does not adsorb allantoin. Pretreated Zeo-Karb, on the other hand, adsorbs 5 to 8 per cent of the allantoin. Zeo-Karb nevertheless may be used if the allantoin found is multiplied by a factor which corrects for the allantoin lost in the column. If the columns are not pretreated with 12 N HCl and are washed instead with dilute  $\text{H}_2\text{SO}_4$ , nearly all of the allantoin as well as the phenylurea, methylurea, and hydantoin is adsorbed.

$$Mg \text{ allantoin per } 100 \text{ cc plasma} = 100 (A_{60} - A_{10})$$

where

$A_{60}$  = the allantoin equivalent of the optical density read after 60 minutes heating,  
 $\lambda$  470  $m\mu$

$A_{10}$  = the allantoin equivalent of the optical density read after 10 minutes heating,  
 $\lambda$  490  $m\mu$

The use of readings taken at wave-length 490  $m\mu$ , on the one hand, and 470  $m\mu$ , on the other, leads to inappreciable error, since both standards and samples are measured the same way and the absorption curve of allantoin over this range is not steep

### *Specificity of Allantoin Method*

The method is not absolutely specific for allantoin. By it any compound can be measured which after treatment with urease in cyanide is not adsorbed by Amberlite or Zeo-Karb H and which gives more yellow to pink color on being heated in acid with diacetyl reagent for 1 hour than is given in 10 minutes heating.

The molecular rings of cyclic derivatives of urea such as alloxan, hydantoin, barbiturates, and to a small extent purines such as caffeine open slowly during prolonged heating in acid and give relatively much more color after being heated 1 hour than after 10 minutes heating. Compounds such as allantoic acid, biuret, and the product resulting from the action of alloxan with cyanide give relatively much color after 10 minutes heating and therefore can contribute in a relatively small way to the lack of specificity of the allantoin method which depends upon the increase in color obtained between the end of the 10 and 60 minute heating intervals.

Methods for the determination of alloxan and evidence of absence of alloxan from normal dog and human plasma are to be given in a later publication. In any case any alloxan present in the sample would have been destroyed during the removal of urea by urease, by the action of the cyanide in which the urease was dissolved. The concentration of barbiturates resulting from medication will seldom if ever, even during a barbiturate anesthesia, reach a level which would interfere with the specificity of the allantoin determination. Uric acid even in the concentrations present in human plasma causes insignificant error. Caffeine yields very little color even after 1 hour's heating but could be a small source of error in plasmas of non-fasting patients.

The fact that slight though appreciable color is formed during the 10 minutes heating of dialysates of urease-treated plasma after treatment with Amberlite or Zeo-Karb leads one to the conclusion that in human and dog plasma there is present some material (other than urea, citrulline,

or allantoin) which gives the carbamido-diacetyl reaction on 10 minutes heating in the phosphoric-sulfuric acid mixture. The intensity of color from this material varies considerably from one plasma to another and is equivalent in the case of human plasma to 15 to 40 per cent of the citrulline present, and to 30 to 80 per cent of the citrulline in the case of dog plasma. The shade of color in both cases is similar to that obtained in the presence of allantoin after more prolonged heating. The presence of this material does not interfere with the specificity of the allantoin (or citrulline) measurement, since allantoin concentration is determined from the difference between the optical densities after 60 minutes and after 10 minutes heating.

It may be noted that the allantoin method of Young *et al* (16) can be used to measure, in addition to allantoin, any allantoic acid present as well as any compound which during the course of the procedure employed by Young would give rise to glyoxylic acid. As pointed out by Christman *et al* (17) the reaction employed in Young's method (16) gives positive results with uric acid and ergothioneine. Since neither of these compounds interferes in the diacetyl method, determination of their concentrations is unnecessary when this method is employed.

### Results

The concentration of citrulline and "allantoin" in a number of blood samples from fasting human subjects is indicated in Table I. Corresponding values on normal fasting dogs under nembutal anesthesia appear in Table II. The 2- to 5-fold rise in citrulline content, the simultaneous marked depletion of circulating arginine, and the consequent depression of urea formation which occur in dogs in severe hemorrhagic shock when *p*-amino hippurate clearance is depressed until it is less than 0.5 per cent of its normal level is to be the subject of a subsequent communication.<sup>10</sup>

The absorption curves of a standard solution of citrulline and of dialysates of dog and human blood plasma are given in Fig. 2. The similarity in shape of the curves for citrulline and for plasma dialysates favors the conclusion that citrulline is present in blood. Amberlite filtrates of plasma dialysates yield essentially the same absorption curve as is given by Zeo-Karb filtrates. The curve given for the dog plasma dialysate is representative of a few in which maximal absorption occurred near  $\lambda$  500 m $\mu$ .

Ormsby (15) has already given the absorption curves of the products formed with urea, allantoin, and citrulline. For comparison, curves given by these and a number of other substances are shown in Fig. 3.

<sup>10</sup> Van Slyke, D. D., Phillips, R. A., Hamilton, P. B., Archibald, R. M., Dole, V. P., and Emerson, K., Jr., unpublished work.

The rates of formation of color on heating dialysates of urease-treated plasmas and solutions of several pure substances under the conditions outlined above are indicated in Figs 4 and 5. For convenience of comparison all readings were made at a wave-length setting of 470  $m\mu$ .

TABLE I  
*Citrulline and Allantoin of Fasting Human Plasma*

Patient	Age	Sex	Diagnosis	Urea clearance	Citrul line*	Allan toin †	$\alpha$ Amino nitrogen by ninhydrin
	yr			per cent of normal	mg per 100 cc	mg per 100 cc	mg per 100 cc
W M	12	M	Normal		0.48	0.3	3.78
S R	14	"	"	130	0.55	0.5	3.27
C S	32	"	"	114	0.48		4.17
R A	34	"	"		0.48		3.96
W G	45	"	"		0.57	0.6	3.80
J D	22	"	"		0.38		3.72
J A	7	F	Healed nephritis	121	0.50	0.62	3.29
M R	5	"	Convalescent nephrotic	113	0.43	0.94	3.02
B W	15	"	Convalescent nephrotic	102	0.73	1.13	3.49
P P	43	"	Essential hypertension	150	0.55	0.91	3.34
L W	5	"	Nephrosis	120	0.35	0.02	3.38
J H	3	M	Acute nephritis	81	0.92	1.6	3.54
A C	37	"	Chronic nephritis	37	0.95		4.5
S S	37	"	" "	79	0.57	0.78	2.99
N O	19	"	" "	6	1.51	3.2	5.70
			uremia	(Blood urea N 121 mg per 100 cc)			
S G	14	"	Chronic nephritis	13	2.02	2.12	3.82
				(Blood urea N 98 mg per 100 cc)			
J Mo	19	"	Malaria		0.82	0.80	
			(chill)		0.47	0.89	
J Me	19	"	"		0.47		
M R	21	"	"		0.60	0.80	

\* These values multiplied by 0.240 give the total citrulline N, by 0.080  $\alpha$ -amino N

† These values multiplied by 0.3544 give total allantoin N

The influence of the concentration and character of the acid used for the diacetyl reaction with citrulline is indicated in Fig 6. In this case all readings were made at 490  $m\mu$  after 10 minutes heating. The abscissae indicate the concentration of acid in 4 cc. to which were added, in all

TABLE II  
Citrulline and Allantoin of Fasting Dog Plasma

Dog	Citrulline	"Allantoin"	$\alpha$ Amino N by ninhydrin
	mg per 100 cc	mg per 100 cc	mg per 100 cc
A	1 20		
B	1 00		
C	1 00		3 45
D	1 28	2 11	
K-67	1 00		
K-68	1 10	1 6	
	1 20		
K-69	0 92	2 6	
	1 01		
	1 55	2 9	
K-70	1 14	3 0	3 66
K-71	0 47	1 1	

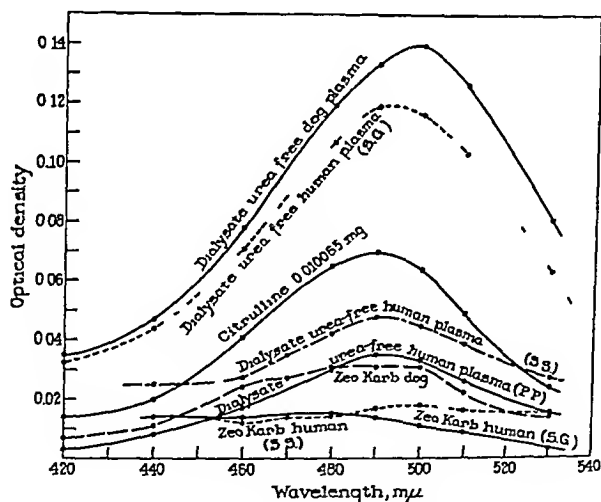


FIG 2 Absorption curve of urea-free dog and human plasma dialysates before and after treatment with Zeo Karb 4 cc of dialysate or Zeo-Karb filtrate (equivalent to 1 cc of plasma) heated 10 minutes at 100° with 2 cc of sulfuric phosphoric acid mixture and 0.25 cc of 3 per cent diacetylmonoxime reagent. Initials of patients are indicated on the curves to enable comparison with the data entered in Table I

cases, 0.1627 mg of citrulline in 2 cc of water and 0.25 cc of the diacetylmonoxime reagent. Since, in the procedure outlined above, only 2 cc of phosphoric-sulfuric acid mixture are used, the point corresponding

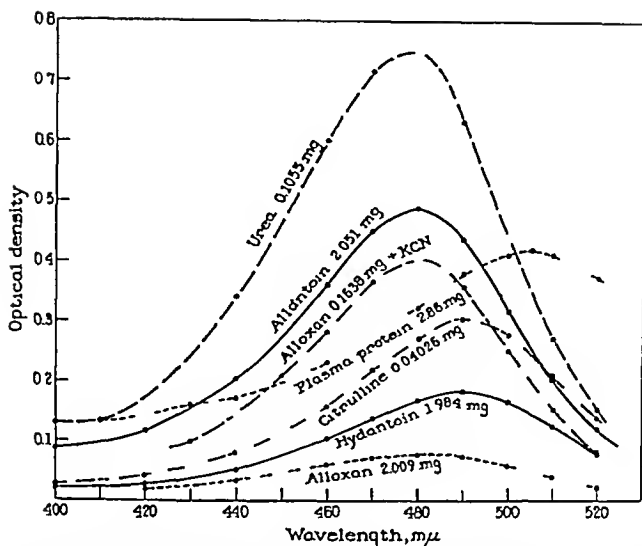


FIG 3 Absorption obtained with substances giving the carbamido diacetyl reaction. The weight indicates the amount present in the 6.25 cc of mixture heated 10 minutes at  $100^\circ$ .

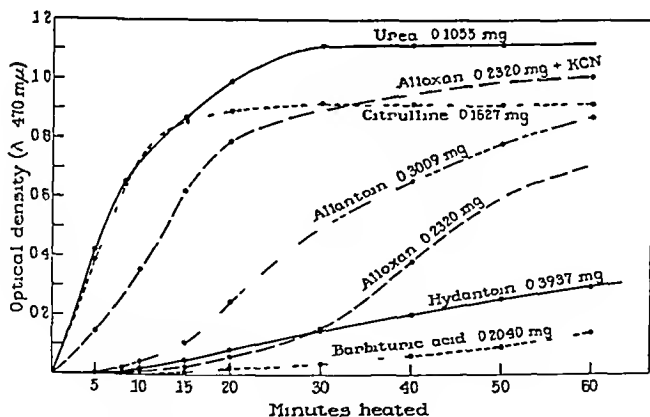


FIG 4 Rate of formation of color at  $100^\circ$  in the carbamido diacetyl reaction with pure substances. Volume 6.25 cc.

to the conditions specified for the citrulline procedure is at  $\times$ . The lighter curves indicate the intensity of color obtained when 0.25 cc of 1 per cent potassium persulfate was added after the heating. After addition of persul-

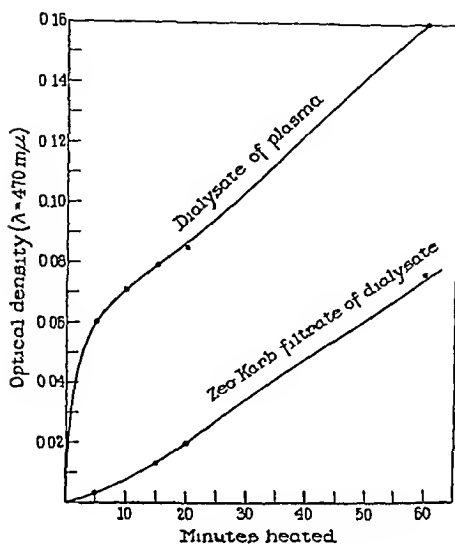


FIG 5 Rate of formation of color at  $100^\circ$  in the carbamido diacetyl reaction, before and after removal of citrulline with Zeo-Karb, from a dialysate of urease treated plasma of a normal fasting dog 4 cc of dialysate or Zeo Karb filtrate in a total volume of 6.25 cc

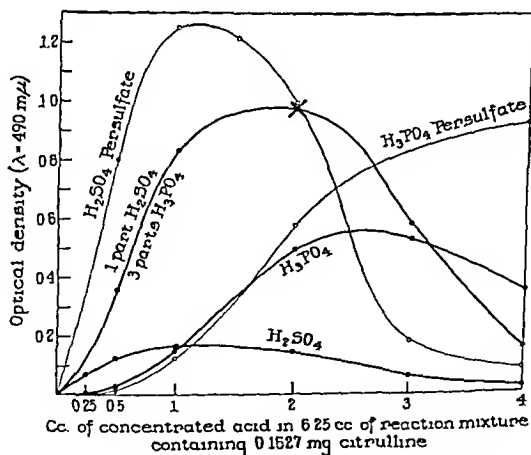


FIG 6 Effect of amount and kind of acid used in the carbamido-diacetyl reaction with citrulline Heated 10 minutes at  $100^\circ$

TABLE III  
*Effect of Light (Diffuse Daylight) on Development and on Stability of Color Obtained with Citrulline*

Citrulline in 6.25 cc solution		Optical density at $\lambda = 490 \text{ m}\mu$																	
		Time standing at room temperature after 10 min heating																	
		5 min		15 min		25 min		60 min		90 min		3 hrs		5 hrs		12 hrs		2 days	
	mg	Dark	Light	Dark	Light	Dark	Light	Dark	Light	Dark	Light	Dark	Light	Dark	Light	Dark	Light	Dark	Light
	0.1627	0.975	0.980	0.975	0.980	0.980	1.08	0.980	1.08	0.980	1.10	1.14	1.10	1.15	1.10	1.10	1.00	1.10	0.37
	0.0813	0.575	0.585	0.619	0.662	0.590	0.704	0.632	0.704	0.628	0.685	0.69	0.450	0.700	0.338	0.72	0.250	0.700	0
	0.03254	0.215	0.222	0.259	0.280	0.238	0.266	0.256	0.266	0.269	0.228	0.30	0.060	0.300	0.034	0.305	0.030	0.271	0
	0.01627	0.089	0.094	0.116	0.116	0.103	0.102	0.126	0.102	0.131	0.072	0.135	0.020	0.130	0.012	0.126	0.018	0.110	0
	0.00814	0.039	0.042	0.048	0.039	0.049	0.027	0.056	0.027	0.058	0.015	0.074	0.010	0.071	0.010	0.009	0.009	0	0



fate the color fades rapidly, especially in the absence of sulfuric acid. The effect of light on the fading of the color obtained in the carbamido-diacetyl reaction in the absence of persulfate and the stability of this color in the dark are indicated in Table III.

Fig 7 indicates the range of concentration over which Beer's law is approximated. Above a concentration of about 0.0005 mm per 6.25 cc of mixture, additional amounts of the urea derivative cause relatively little increase in color. Curves obtained with methylurea when double and half the usual amounts of diacetylmonoxime reagent in the same vol

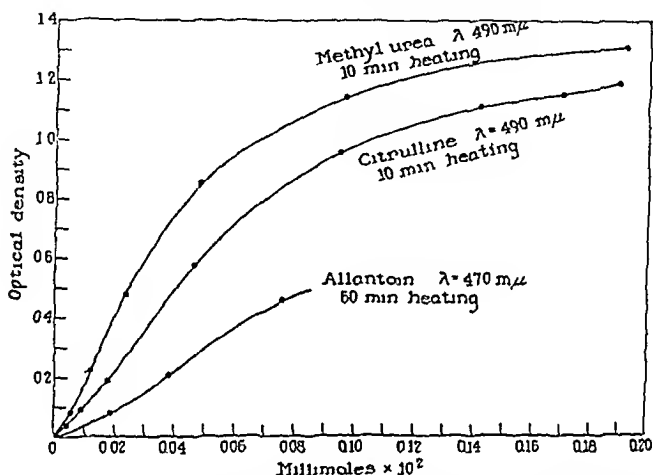


FIG 7 Relation between concentration of the carbamido compound and amount of color formed in the reaction with diacetylmonoxime. Conditions were as prescribed in the procedure for analysis.

ume were used were identical with the methylurea curve presented in Fig 7. Neither prolongation of the heating period nor addition of persulfate solution after the heating changed the shape of the curve. Therefore the factor limiting color production appears to be neither the concentration of diacetylmonoxime or of the oxidant nor the duration of the heating period.

### Discussion of Results

Both citrulline and allantoin when added to plasma have been "recovered" by the above procedure with an error of less than  $\pm 5$  per cent. This by itself does not mean, of course, that the material measured in either fraction is entirely citrulline or entirely allantoin. Christman *et al* (17) have recently applied the principle of Young's method to the

measurement of allantoin in human blood After correcting for the uric acid and ergothioneine present, they obtained a negative value for allantoin Similar negative values were obtained with whole horse blood, although results obtained with horse plasma indicated the presence of allantoin At present there is insufficient evidence to indicate whether an overcorrection by Christman has led him to miss allantoin present in human plasma or whether the positive allantoin figures given by the method here described include "allantoin-like" substance In any case the present method, unlike that of Christman, does not give positive results with either uric acid or thioneine It is probable that the values given by this method for plasma allantoin are somewhat too high

The curves presented were obtained with a Coleman junior spectrophotometer The location of each absorption maximum was verified by using the Coleman spectrophotometer No 10S For convenience of reference and comparison of results a constant for the cuvette used is given

A solution of  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  M/80 in 2 N  $\text{NH}_4\text{OH}$  read at a wave-length of 620  $\text{m}\mu$  against 2 N  $\text{NH}_4\text{OH}$  had an optical density of 1.15 According to Drabkin and Austin (18) the extinction coefficient of M/80 copper ammonium sulfate under these conditions is 58

The author wishes to acknowledge the helpful suggestions of Dr D D Van Slyke in whose laboratory the work was conducted, the help of Dr R A Phillips and Dr P B Hamilton in obtaining specimens of dog blood, and the technical assistance of Miss E Stroh and Miss P Ortiz

#### SUMMARY

1 A method for the determination of citrulline in plasma is outlined The method has been applied also to the determination of citrulline in dialysates of enzymatic digests of proteins

2 The specificity of the carbamido-diacetyl reaction has been investigated further and its usefulness extended by including a fractionation with Amberlite or Zeo-Karb H

3 Evidence is presented which indicates that the normal fasting plasma level is between 0.3 and 1.0 mg of citrulline per 100 cc for man and 0.8 to 1.5 mg per 100 cc for the dog

4 A simple method for the determination of allantoin in plasma is included This method is uninfluenced by the presence of uric acid or thioneine, but is not entirely specific for allantoin

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# THE RIBONUCLEASE ACTIVITY OF PASTEURELLA PESTIS (PLAGUE BACILLUS)

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Living *Pasteurella pestis* cells, cells killed by phenylmercuric nitrate, and a cell-free preparation are shown in this report to decompose the ribonucleic acid of yeast. The predominant action appears to be depolymerization. Tetranucleotidase action takes place to a smaller extent, mononucleotides being formed, but decomposition beyond this stage is very slight. The enzymes are somewhat resistant to heat, the greatest stability of the depolymerase being found at pH 6.5 and of the tetranucleotidase at pH 7.6.

The enzyme activity found in *Pasteurella pestis* appears to be similar to that of the ribonuclease of pancreas which was discovered by Jones (1) and recently prepared in crystalline form by Kunitz (2). Schmidt and Levene (3) considered that the function of the pancreas enzyme was limited to depolymerization, since they found no evidence of mononucleotide formation. However, Loring and Carpenter (4) have isolated the four mononucleotides after digestion of ribonucleic acid by the crystalline enzyme of Kunitz. Although ribonuclease has been found in largest amount in pancreas, it has been observed in other tissues (5-7).

The criterion for measuring enzyme activity has been in the past the amount of nucleic acid phosphorus rendered soluble in acid or in the uranium reagent of MacFadyen (8), without distinction between the two reagents. It appears, however, that considerably more enzyme-digested nucleic acid remains soluble when precipitated by HCl than when precipitated by uranium. This is true when the digestion is brought about by the pancreas enzyme (5) as well as by the *Pasteurella pestis* enzyme. Acid and uranium therefore measure different stages of digestion of the nucleic acid. Since the uranium reagent does not precipitate any of the four mononucleotides (8), it must be assumed that it is precipitating nucleic acid in a lower stage of depolymerization than is precipitated by HCl, probably the simple tetranucleotide, although this has not been available for testing. The phosphorus which becomes soluble in the uranium reagent would be therefore a measure of hydrolysis of tetranucleotide (tetranucleotidase action), while that which becomes soluble in HCl would be a measure of depolymerization.

## EXPERIMENTAL

*Pasteurella pestis* Preparations—The avirulent strains of *Pasteurella pestis*, Soemedang and Tjwidej, were grown at 27° for 2 days in 1000 cc. of

Casamino acid medium containing 0.5 per cent glucose as described by Smith (9). If the living cells were to be used, the culture was centrifuged under aseptic conditions, the sedimented cells washed by centrifuging, and then resuspended in a small volume of water. If killed cells were to be used, phenylmercuric nitrate was added to the culture to make a concentration of 1:40,000, and allowed to act upon the culture 2 hours before centrifuging. The cell-free preparation was made from the cells killed by phenylmercuric nitrate. The cell suspension (10 cc.) was submitted to intense sonic oscillation (frequency about 10,000 cycles per second) for 20 minutes, which broke up the cells. By centrifuging in a multispeed centrifuge, a slightly milky cell-free supernatant fluid was obtained.

*Purification of Nucleic Acid*—We attempted to purify the nucleic acid<sup>1</sup> by glacial acetic acid precipitation according to the procedure described by Kunitz (2). The procedure had to be modified, however, since a gummy mass was obtained when water was added for washing. If acetic acid (5 parts of glacial acetic acid to 1 part of water) was used for washing, the material remained powdery and washing was facilitated. The washing was then completed with alcohol and ether. The cake when dried in air was easily broken up, yielding a white powder. The material was dried further in a vacuum over NaOH.

The nucleic acid, so purified, gave an analysis of 14.0 per cent nitrogen and 8.2 per cent phosphorus. The purified product contains considerably more of a higher polymer than the crude acid. This is shown by the fact that only 7 per cent of the total phosphorus of the purified product remained soluble in HCl when precipitated from 1 per cent solution (see the next section) as compared to 38 per cent for the crude product, while the corresponding figures were 1 and 12 per cent when the solution was precipitated by the uranium reagent. The almost complete insolubility in the uranium reagent indicated that no molecule smaller than a tetranucleotide was present in the purified product.

*Hydrochloric Acid Precipitation of Nucleic Acid*—In the experiments of Schmidt and Levene (3) the proportion of nucleic acid phosphorus remaining soluble on precipitation by hydrochloric acid depended upon the concentration of nucleic acid at precipitation. This is true, also, of the purified nucleic acid described above, although the solubility has been exceedingly diminished by the purification. Data obtained when 1 cc. of 0.5 N HCl was added to 1 cc. of nucleic acid solution are given in Table I. Precipitation was carried out at both 20° and 5° for comparison. The supernatant fluid obtained by centrifuging at room temperature was analyzed for total nitrogen and total phosphorus.

The data show that, with both the purified and crude nucleic acid, the

<sup>1</sup> Pfanstiehl nucleic acid, from yeast

percentage of total N or P remaining soluble increased as the concentration of nucleic acid decreased. This was true with the purified acid, whether the precipitation was made at 20° or at 5°. With the purified acid, there was not a great difference in the solubility at 20° and at 5°, while with the crude acid there was a large difference. This suggests that the crude acid contains more of a lower polymer of the tetranucleotide which will precipitate to a greater extent at 5° than at 20°. Since, in the experiments on digestion of nucleic acid, a measure of conversion of higher polymer was desired, a precipitation temperature of 20° has been adopted, and a concentration of nucleic acid as nearly 1 per cent as possible has been maintained.

*Hydrochloric Acid Turbidity Method for Nucleic Acid Measurement*—A convenient method for detecting the first changes in nucleic acid produced by the depolymerase action was found in measurement of the turbidity

TABLE I  
*Solubility of Nucleic Acid in 0.25 N HCl*

Nucleic acid concentration	Purified nucleic acid				Crude nucleic acid	
	Soluble N		Soluble P		Soluble P	
	20	5	20	5	20	5
<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
4.0					21.8	
2.0					28.4	
1.0	7.0	6.2	6.9	5.5	38.2	21.6
0.5	10.0	7.3	8.1	6.9	41.8	
0.2	13.1	10.7		9.1		
0.1	20.1	15.1		12.1		

produced at 25° when 1 cc. of 0.5 N HCl was added to 1 cc. of very dilute solutions of nucleic acid (0.05 to 0.5 mg.). The particles were so finely divided that no settling occurred and the turbidity remained constant for sufficient time for reproducible readings. A standard curve was set up by measuring in a photoelectric colorimeter the turbidity produced by different amounts of the original nucleic acid and plotting on semilogarithmic paper. From the turbidity produced by the nucleic acid in a digested sample, the amount of unchanged nucleic acid may be read directly from the standard curve.

*Digestion of Nucleic Acid by Living Cells of Pasteurella pestis*—A series of tubes, each containing 1 cc. of a suspension of living *Pasteurella pestis* cells of the Soemedang strain and 5 cc. of 4 per cent nucleic acid solution adjusted to pH 7.5 with NaOH, was made up under aseptic conditions. Corresponding control tubes were made similarly, in which either the nucleic acid or the cell suspension was replaced with water. The tubes were incubated at

37°, one tube of each set being removed at time intervals for analysis. To prepare a tube for analysis it was first heated in a water bath at 80–90° for 10 minutes to kill the bacterial cells. After cooling, the cell suspension was centrifuged and the supernatant solution used.

By the turbidimetric method it was shown that nucleic acid disappeared only in the presence of the cells. The data, Table II, (a), show that the loss of nucleic acid increased with time of incubation. At the same time there was a gradual, but very small, liberation of free phosphate. At the end of 7 days incubation, the digestion mixture was analyzed for purine by Bloch's modification (10) of the Graff and Maculla method (11), and for reducing sugar by the Hagedorn-Jensen method (12). No free purine or sugar was found, and there had been no loss of total purine or sugar, as indicated by

TABLE II  
*Digestion of Nucleic Acid by Pasteurella pestis*

	Time of incubation	Nucleic acid loss (turbidimetric method)	Inorganic P liberation
	days	per cent	per cent
(a) Living cells	1	9	0.5
	3	29	1.2
	7	48	2.1
(b) Cells killed by phenylmercuric nitrate*	1	18.5	0.2
	4	43.8	0.5
	7	63.5	1.0
(c) Cell-free preparation*	1	13.2	0.4
	4	58.1	0.7
	7	65.2	1.0

\* Same culture used for (b) and (c)

the values after acid hydrolysis. The decomposition of nucleic acid, therefore, had not proceeded appreciably beyond the mononucleotide stage.

A similar experiment carried out at pH 6.7 and continued for 21 days showed 89 per cent loss of nucleic acid with the liberation of only 4.7 per cent of the total phosphorus.

*Comparison of Digestion of Nucleic Acid by Intact Killed Cells of Pasteurella pestis and by a Cell-Free Preparation*—Parallel experiments, in which a suspension of cells of the Tjwidej strain killed by phenylmercuric nitrate or of the cell-free preparation from these containing the intracellular constituents in corresponding concentration was used, gave the data in Table II, (b) and (c). This shows that the killed cells have the same enzymatic effect upon nucleic acid as the living cells, and that essentially all of the enzymatic activity is present in the cell-free preparation. Similar results were obtained when the Soemedang strain of *Pasteurella pestis* was used.

*Nature of Digestion of Nucleic Acid by Intracellular Constituents of Pasteurella pestis*—A digestion mixture was made containing 2.5 cc of cell-free *Pasteurella pestis* preparation, made from the Soemedang strain, and 10

TABLE III

*Products of Digestion of Yeast Nucleic Acid by Pasteurella pestis at pH 6.5*

Analysis of 1 cc of incubation mixture containing 10 mg of nucleic acid (1.407 mg of N, 0.822 mg of P)

Incubation	Depolymerization			Hydrolysis beyond tetranucleotide						Mononucleotide formation		Inorganic $\text{PO}_4$ formation	
	Analysis of HCl supernatant			Analysis of uranium fractions						Analysis of Pb ppt. N found	Mononucleotide formed	P found	P liberated
				N found		Tetranucleotide lost	P found		Tetranucleotide lost				
	N found	P found	Nucleic acid lost	Ppt	Supernatant		Ppt	Supernatant					

Nucleic acid and *P. pestis*

days	mg	mg	per cent* from N	per cent* from P	mg	mg	per cent†	mg	mg	per cent†	mg	per cent†	mg	per cent†
0	0.168	0.083			1.575	0.049		0.844	0.023		0.021		0.009	
3	0.902	0.494	57	56	1.295	0.310	20	0.697	0.167	18	0.245	16	0.021	1.5
7	1.182	0.692	79	82	1.170	0.440	30	0.623	0.247	28	0.378	26	0.030	2.6

## Nucleic acid control

0	0.124	0.081			1.387	0.035		0.812	0.018		0.021		0.006	
3	0.128	0.086	0.3	0.7	1.345	0.050	0	0.788	0.020	0	0.021	0	0.004	0
7	0.128	0.095	0.3	1.9	1.370	0.035	0	0.805	0.022	0	0.021	0	0.005	0
Average	0.126				1.367	0.040		0.802	0.020		0.021		0.005	

*P. pestis* control

0	0.185	0.032			0.161	0.014		0.028	0.005	0			0.003	
3	0.174	0.036			0.165	0.035		0.027	0.007	0			0.004	
7	0.179	0.034			0.189	0.021		0.028	0.007	0			0.005	
Average	0.179	0.034			0.172	0.023		0.028	0.006				0.004	

$$* \frac{\text{Increase in N or P}}{1.407 - 0.126 \text{ (for N) or } 0.822 - 0.081 \text{ (for P)}} \times 100$$

$$\dagger \frac{\text{Increase or decrease in N or P}}{1.367 \text{ (for N) or } 0.802 \text{ (for P)}} \times 100$$

cc of 1.25 per cent nucleic acid (dissolved in the cold by enough NaOH to bring the pH up to 6.6). The resulting pH of the digestion mixture was 6.5. Nucleic acid and *Pasteurella pestis* controls were made by replacing



the cell-free preparation or the nucleic acid, respectively, with water. The mixtures were incubated at 37° after a few drops of toluene were added to prevent bacterial infection. Immediately after the mixing, and after 3 and 7 days of incubation, samples were removed from each mixture for HCl and uranium precipitation.

HCl precipitation was made as described in a preceding section. Uranium and lead fractionation was carried out as described by MacFadyen (8). The fractions were analyzed for total N by the micro-Kjeldahl method, and for total P by the method of King (13), sulfuric acid and hydrogen peroxide being used for digestion. Inorganic phosphate present was determined in the supernatant fluid from the uranium precipitation.

The analytical data obtained, Table III, show that there was practically no change in either the nucleic acid control or the *Pasteurella pestis* control.

TABLE IV

*Ribonuclease Activity of Cell-Free Pasteurella pestis Preparation after Heating at 90° for 10 Minutes*

pH during heating	Depolymerase	Tetranucleotidase	Mononucleotidase
	Amount of P soluble in HCl	Amount of P not pptd by uranium reagent	Amount of inorganic P liberated
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
4.9	16.5	5.5	0.20
6.5	23.7	9.8	0.22
7.6	27.5	11.1	0.49
8.7	24.0	8.9	0.33
Unheated control	37.5	14.7	0.92

during the extent of the experiment. In the mixture of nucleic acid and *Pasteurella pestis* there was a large increase in the amount of acid-soluble N or P as the time of incubation increased, and a smaller increase in the amount of N or P which remained soluble in the uranium reagent. These data indicate that a much greater amount of nucleic acid has been changed from its original state than has been decomposed beyond the tetranucleotide stage. The course of enzyme action, therefore, appears to be depolymerization followed by hydrolysis of tetranucleotide. The mononucleotide formation, shown by nitrogen analysis of the lead precipitate, accounted for almost all of the tetranucleotide units lost. The *Pasteurella pestis* enzymes, therefore, show very little mononucleotidase action, an observation which is confirmed by the very small amount of inorganic phosphate which is liberated.

*Effect of Heat on Stability of Pasteurella pestis Ribonuclease*—Tubes containing 1 cc. of cell-free *Pasteurella pestis* preparation from the Soemedang

stram, adjusted to different conditions of pH by HCl or NaOH, were heated in a water bath at 90° for 10 minutes. All the solutions were then re-adjusted to the original pH, 6.5, before nucleic acid was added for measurement of the enzyme activity. The decomposition of nucleic acid during a digestion period of 5 days at 37° produced by the heat-treated enzyme preparations is shown in Table IV. It will be seen that a part of the depolymerase, tetranucleotidase, and mononucleotidase activities has been destroyed at each pH studied. The least destruction of depolymerase was at pH 6.5, and of tetranucleotidase and mononucleotidase at pH 7.6. There has been relatively more loss of mononucleotidase than of tetranucleotidase or depolymerase.

#### SUMMARY

Analytical data, obtained from hydrochloric acid precipitation and uranium fractionation, show that yeast nucleic acid is enzymatically decomposed by living cells of *Pasteurella pestis*, cells killed by phenylmercuric nitrate and by a cell-free preparation. Only part of the nucleic acid decomposed is hydrolyzed to mononucleotides, the remainder probably existing in a depolymerized state. The decomposition is accompanied by liberation of only a trace of inorganic phosphate.

All of the enzymes of the ribonuclease system are inactivated somewhat by heat, the least inactivation of the depolymerase being produced at pH 6.5 and of the tetranucleotidase and mononucleotidase at pH 7.6.

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# PHOSPHOLIPID TURNOVER FOLLOWING ADMINISTRATION OF DIETHYLSTILBESTROL TO COCKS

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The concentration of plasma phospholipids increases markedly in birds after the administration of natural estrogen or diethylstilbestrol (1, 2). The increase occurs without a comparable decrease of the phospholipid content of the liver, which is generally considered to be the site of formation of the plasma phospholipids (3-6). The increased concentration in the plasma might be due either to an increased synthesis in the liver or to an inhibition of destruction of phospholipid through estrogen action as suggested by Burr and Barnes (7). These possibilities have been investigated with radioactive phosphorus. This has been administered as inorganic phosphate to cocks after the administration of diethylstilbestrol and the uptake of the  $P^{32}$  in phospholipids of plasma and liver has been compared with that in normal birds. Also comparisons were made on the rate of disappearance of administered radioactive phospholipid in treated and untreated birds.

## Methods

White Leghorn cocks weighing from 1.8 to 2.5 kilos, maintained in cages on a standard ration, were studied in May, 1943, and from November, 1943, to April, 1944. Diethylstilbestrol dissolved in peanut oil was injected intramuscularly at the times indicated in the various experiments, each injection represented 1 mg. of diethylstilbestrol for each kilo of body weight of the bird used. 20 microcuries of radioactive phosphorus for each kilo of body weight was injected intraperitoneally in aqueous solution of 1 to 2 mg. of dibasic sodium phosphate. We are greatly indebted to Dr. Carl Helmholtz and Dr. John Lawrence of the Radiation Laboratory, University of California, Berkeley, for the radioactive phosphate used in these experiments.

Samples of blood were removed by venipuncture and the birds were anesthetized with intravenously administered phenobarbital sodium just prior to removal of the liver. After extraction with trichloroacetic acid, the heparinized plasma was analyzed photometrically by the method of Fiske and Subbarow (8) for concentration of inorganic phosphate, and the radioactivity of this was measured by a Geiger-Muller counter of the immersion type (9). An aliquot of plasma was extracted for phospholipids with 20

volumes of a 3:1 alcohol-ether mixture at room temperature for 1 hour or longer. An aliquot of the extract was ashed with sulfuric acid and superoxol and the phosphorus content was determined. Another aliquot was used for measurement of radioactivity. Zero values for radioactivity of the phospholipids were consistently found 15 minutes after the administration of the radioactive phosphorus, at which time the radioactivity of the inorganic phosphate was very high. This indicates the completeness of separation of phospholipid from the inorganic phosphate with the simple procedure used.

After a portion of the liver had been ground with sand, the phospholipids of the liver were extracted with alcohol for 1 hour and then with ether for two 1 hour periods in a Bailey-Walker extractor. After the addition of a small quantity of hydroquinone, the extract was evaporated to dryness by aeration at room temperature. The residue was dissolved in chloroform. An aliquot was ashed and the phosphorus content determined. The radioactivity of another aliquot was measured.

Another portion of the liver was quickly frozen with carbon dioxide ice and alcohol and extracted with 5 per cent trichloroacetic acid. The inorganic phosphate was precipitated from an aliquot as the magnesium ammonium salt. Another aliquot was fractionated with barium hydroxide. From the barium-soluble fraction the barium salt of glycerophosphoric acid was precipitated with 5 volumes of ammoniacal alcohol. Measurements of phosphorus and radioactivity were made and counts per second per mg P were calculated.

### Results

In a preliminary experiment three cocks received  $P^{32}$  and diethylstilbestrol on the 2nd, 3rd, and 4th day of daily treatment with the estrogen. The amount and radioactivity of the phospholipid of the blood of each were determined at intervals for the next 24 hours. Only a small amount of radioactivity was found in the phospholipid of the plasma 2 hours after injection, then followed a rapid increase up to 12 hours and a small decrease at 24 hours (Fig. 1). The specific activity of the plasma phospholipids was similar in the three birds. The total amount of labeled phospholipid in the plasma increased with the phospholipid content of the plasma, which was 36.9, 81.2, and 96.2 mg of phospholipid P in 100 ml of plasma at the 24 hour period. The plasma of Cocks 2 and 3 contained 2.74 and 2.80 times as much radioactive phospholipid as that of Cock 1.

Analyses were made on the plasma and liver 6 hours after the administration of  $P^{32}$  in sixteen untreated cocks and sixteen which had received diethylstilbestrol daily, the first injection having been given from 18 to 126 hours before the termination of the experiment. The essential data from

these birds are abstracted in Tables I and II. The amount of inorganic phosphate, phospholipid, and glycerophosphate in the liver was almost identical in the birds that received diethylstilbestrol and in those that were untreated. There was a wide variation in both groups of birds in the specific activity of the inorganic phosphate of the plasma and liver. Similar variation occurred in the specific and relative activities of the phospholipid of the plasma and liver as well as the glycerophosphate of the liver. The mean values of the specific and relative activities of these substances

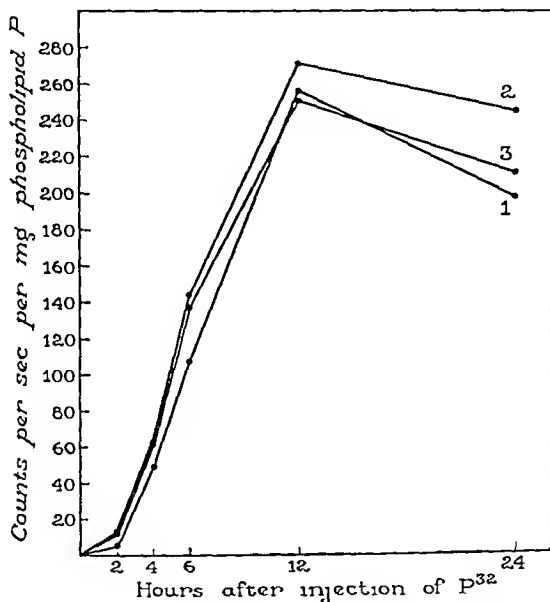


FIG 1 Specific activity of phospholipid of plasma after injection of  $P^{32}$ . Each bird received diethylstilbestrol at the same time as the radioactive sodium phosphate. Cock 1 had received one injection of the estrogen 24 hours earlier, Cock 2 two previous injections at 24 hour intervals, and Cock 3 three injections at 24 hour intervals.

did not show any significant differences between the treated and the untreated birds. Because of the increased amount of phospholipid in the plasma of the treated birds the mean value of the total radioactive phospholipid in the blood was  $991 \pm 145$  counts per second for each 100 ml of plasma and  $329 \pm 64$  in the untreated birds. 3 times as much labeled phospholipid had entered the plasma of the treated birds as had entered the plasma of those not receiving diethylstilbestrol.

In the treated birds the mean value for the relative activity of the mor-

ganic phosphate of the liver was not significantly greater than that of the plasma, and in the untreated birds it was almost the same as that of the plasma. The relative activity of the glycerophosphate of the liver of both groups was not greatly different from that of the inorganic phosphate and about twice that of the phospholipid of the liver. The relative activity of the phospholipid of the liver was greater than that of the phospholipid of

TABLE I  
*Distribution of P<sup>32</sup> in Plasma 6 Hours after Injection\**

Inorganic phosphate				Phospholipid				Treated birds Hrs after first injection of diethylstilbestrol
Mg P in 100 ml		Specific activity		Mg P in 100 ml		Relative activity		
Untreated	Treated	Untreated	Treated	Untreated	Treated	Untreated	Treated	
4 86	5 06	220	441	6 06	10 06	68	2	18
3 00	4 90	385	282	6 31	11 44	34	103	18
2 83	6 06	328	323	6 43	11 75	44	30	30
4 50	3 96	261	389	6 50	17 00	38	40	54
4 56	6 93	264	167	6 81	18 13	59	59	30
4 33	5 93	335	242	6 81	19 06	36	42	30
2 16	4 50	738	443	6 90	19 75	21	25	54
4 03	5 63	341	321	7 06	24 75	54	40	30
3 90	2 90	344	402	7 20	32 70	28	48	54
2 66	6 47	453	536	7 45	39 60	15	29	54
3 43	4 60	342	608	7 69	46 50	37	15	54
2 93	5 27	601	549	8 25	48 00	19	14	54
3 00	6 53	261	522	9 25	61 25	52	28	54
4 00	7 46	510	454	9 38	64 70	18	8	126
2 93	6 67	369	534	9 38	77 25	35	28	54
4 30	9 33	337	367	17 25	81 25	70	22	126
3 59	5 76	380	411	8 04	36 45	39	33	
±0 2	±0 38	±34	±31	±0 67	±6 0	±4	±6	

\* Specific activity is expressed in terms of counts each second for each mg of P. Relative activity is specific activity  $\times$  100 divided by the specific activity of the plasma inorganic phosphate. The values after the  $\pm$  sign represent the standard error of the mean. The data obtained from both untreated and treated birds are tabulated according to the amount of phospholipid in the plasma.

the plasma except in four of the thirty-two birds, in which it was slightly less. The calculations of the amount of phospholipid entering the plasma from the liver will be discussed later.

Radioactive phospholipid was synthesized by two donor cocks and their plasma containing this was injected into four untreated cocks and four cocks treated with diethylstilbestrol. The production of phospholipids in the donor cocks was stimulated by injections of 1 mg of diethylstilbestrol.

per kilo given once a day for 4 days and at 12 hour intervals on the 5th day 40 microcuries of  $P^{32}$  were administered 12 hours prior to removal of the

TABLE II  
*Distribution of  $P^{32}$  in Liver 6 Hours after Injection\**

Relative activities						Phospholipid from liver in plasma	
Inorganic phosphate		Glycerophosphate		Phospholipid		Mg P in 100 ml	
Untreated	Treated	Untreated	Treated	Untreated	Treated	Untreated	Treated
129	80	113	35	54	10	7.7	5.0
168	84	157	97	58	72	3.7	16.4
151	119	128	101	60	51	4.7	6.9
149	117	123	115	66	66	3.8	10.6
73	139	100	138	56	69	7.2	16.5
124	127	109	122	43	54	5.7	14.8
66	96	63	89	32	38	4.5	13.0
110	110	80	104	48	49	7.9	20.1
135	83	104	84	40	52	5.0	30.1
126	75	89	82	34	49	3.3	23.4
134	88	102	83	55	33	5.1	21.2
130	129	88	92	28	32	5.6	21.0
180	70	170	64	71	54	6.8	31.8
109	122	81	73	28	36	6.0	14.8
123	57	110	64	42	53	7.8	40.8
145	89	128	75	88	54	13.7	33.1
128 ± 7	99 ± 6	109 ± 7	88 ± 6	50 ± 4	48 ± 4	6.2 ± 0.6	20.0 ± 2.5

\* Relative activity is the specific activity  $\times 100$  divided by the specific activity of the inorganic phosphate of the plasma. The values after the  $\pm$  sign represent the standard error of the mean. The data of Table II are arranged in the same order as those of Table I.

TABLE III  
*Composition of Donor's Plasma*

Donor No	Inorganic phosphate		Phospholipid	
	mg P in 100 ml	counts per mg P	mg P in 100 ml	counts per mg P
1	7.50	780	56.12	193
2	5.80	881	54.50	417

plasma. The concentration of phospholipid was elevated in these cocks (Table III).

8 ml for each kilo of body weight of plasma from Donor 1, containing 3.5 mg of phospholipid P with 1736 counts per second and 0.38 mg of in-



organic phosphate P with 468 counts, was given intravenously to four recipient cocks 5 ml of plasma from Donor 2, containing 2.7 mg of phospholipid P with 1140 counts and 0.29 mg of inorganic phosphate P with 255 counts, was given to four additional recipients. The weights of the recipient cocks varied from 1.88 to 2.57 kilos. Two of the recipients from each donor were untreated cocks, two had received injections of 1 mg of stilbestrol per kilo 48, 24, and 12 hours before the administration of the labeled phospholipids and thus had elevated concentrations of plasma phospholipids.

Samples of blood were removed for analyses at 15 minutes, 1, 3, and 6 hours after the administration of the radioactive phospholipid. The total concentration of plasma phospholipids decreased during this interval in the untreated cocks but either remained approximately at the original level or increased in the treated cocks, owing to the continued action of the estrogen.

TABLE IV

*Phospholipid of Plasma after Intravenous Injection of Radioactive Phospholipid*

Treatment	Inorganic phosphate		Phospholipid			
	0.25 hr	6 hrs	0.25 hr	6 hrs	0.25 hr	6 hrs
	counts per sec per ml	counts per sec per ml	mg P in 100 ml	mg P in 100 ml	counts per sec per ml	counts per sec per ml
None	8	6	24.8	11.0	38	14
"	9	6	17.6	13.2	43	16
"	4	2	14.1	8.3	28	9
"	5	3	13.1	9.8	26	11
Diethylstilbestrol	8	5	33.9	38.8	48	23
"	7	4	35.8	34.4	41	24
"	5	1	25.3	23.2	29	11
"	4	1	28.2	31.1	26	13

(Table IV) In 5.75 hours after the first mixed specimen was taken the concentration of radioactive phospholipid decreased 63, 63, 68, and 58 per cent in the plasma of the untreated birds and 52, 42, 62, and 50 per cent in the treated birds. With the assumption that the unlabeled phospholipid left the blood at the same rate as that labeled, this indicates that 15.6, 11.1, 9.5, and 7.6 mg of phospholipid P left each 100 ml of plasma in 5.75 hours in the untreated birds and that 17.6, 14.9, 16.7, and 14.1 mg left the plasma of the birds which had elevated plasma phospholipid values because of administration of diethylstilbestrol.

### Comment

The observation of an increased total radioactivity of plasma phospholipids in cocks treated with diethylstilbestrol compared with untreated cocks is in line with the findings of Entenman and his associates (10) of more

labeled phospholipid in the plasma at 6 and 12 hours after the administration of  $P^{32}$  in laying than in non-laying hens. It does indicate that an increased synthesis of plasma phospholipid occurs in cocks after treatment with diethylstilbestrol. The difference between the mean relative specific activities of the plasma phospholipids in the treated and untreated cocks was small. This finding is in keeping with the obvious fact that the newly formed phospholipid enters the plasma at about the same rate as phospholipid leaves the blood. In the birds that received diethylstilbestrol there was only a small increase in the phospholipid content of the blood during the period of 6 hours that labeled phospholipid was being formed. The similarity of the ratio of labeled to non-labeled phospholipid in both groups of birds suggests a more rapid turnover of phospholipid in the blood of the treated birds having a larger amount of phospholipid in their blood than in the blood of the untreated birds.

The relative specific activity of the hepatic phospholipid was greater than that of the plasma phospholipid. Such a relationship has been found by Hevesy and his associates (3, 4), Artom (5), and Fishler and his associates (6), and this has been considered as indirect evidence that the liver is the main site of formation of plasma phospholipids. More direct evidence for this is found in experiments of Fishler and his associates (6), who have administered  $P^{32}$  to hepatectomized dogs and observed up to 6 hours later only negligible amounts of labeled phospholipid in the plasma, although the concentration in the kidneys and in the small intestine attained the same levels as in intact dogs.

The mean relative specific activities in the untreated cocks and in those treated with diethylstilbestrol were not significantly different. Entenman and his associates (10) did not find an increase in labeled hepatic phospholipids associated with the increase in labeled plasma phospholipids found in laying hens. The liver apparently increases the output of phospholipids into the plasma as its production of phospholipid increases.

The administered inorganic phosphate enters the liver rapidly but whether it enters into synthesis of phospholipid in the liver as readily is not known. Fishler and his associates (11) did not observe any decrease in the amount of labeled phospholipid in slices of liver maintained for 1 hour in bicarbonate-Ringer's solution and concluded that a rapid enzymatic action involving inorganic phosphate and phospholipid does not occur. Glycerophosphate has about as high a specific activity as does the inorganic phosphate of the liver and therefore might serve equally well as a precursor of the phospholipids. In fact Taurog *et al* (12) found that glycerophosphate was incorporated into phospholipid of liver both *in vitro* and *in vivo*. However, the possibility of preliminary hydrolysis to inorganic phosphate was not excluded. The plasma of birds treated with diethylstilbestrol becomes very lipemic and the increase in available fat may affect the rate of

synthesis of the phospholipids. Thus Hahn and Hevesy (13) found an increase in the amount of labeled phospholipid in perfused livers with an increase in the fat content of the perfusing blood. Whether the cellular oxidations which are essential for the formation of phospholipids (12) limit the rate of synthesis is not known. Difficulties involved in the calculations of the rates of synthesis of phospholipids from data on the specific activity of plasma have been discussed by Chaikoff (14) and by Zilversmit and his associates (15). The variability in the specific activity of the plasma inorganic phosphate and the concentration of plasma phospholipids, and thus in the output of phospholipids by the liver during the 6 hour interval studied in our experiments, would complicate such calculations considerably.

However, such calculations from the data that we have appear to be warranted and the results of the calculations indicate that a close approximation to the number of mg of phospholipid transferred from the liver to the blood may be obtained. The rate of increase of radioactive phospholipid in the plasma after administration of radioactive phosphate indicates that only a slight amount is added in the first 2 hours but that after that time and for the next 10 hours the rate of increase is constant (Fig 1). This fact suggests that the radioactivity of the incoming phospholipid is constant, since there is no reason to assume that phospholipid of greater activity is entering the plasma at a reduced rate. The liver is the source of almost all, if not all, of the phospholipid of the plasma and, although not all of the phospholipids of the liver may enter the plasma, the relative activity of that entering the plasma is probably not greatly different from that of the entire phospholipids of the liver at the end of 6 hours. For example, 6 hours after injection of  $P^{32}$  the plasma phospholipid P was 6.31 mg with a relative activity of 34 and the relative activity of the phospholipid of the liver was 58. 3.7 mg of phospholipid P of activity 58 are present in 6.31 mg with activity of 34.

The calculations of the phospholipid from the liver in the plasma shown in Table II are on this basis. The results are uniform in the untreated birds, indicating that  $6.2 \pm 0.6$  mg of phospholipid P was added to the plasma in 4 hours. In the treated birds greater amounts were added in the birds having more total phospholipid in the plasma with a mean value of  $20.0 \pm 2.5$  for the amount added in 4 hours. This indicates a complete replacement of an amount of phospholipid equivalent to the amount in normal blood in 5.3 hours and a somewhat longer time for complete replacement when the phospholipid content of the blood is elevated, although the amount added is considerably larger than in the normal. Approximately 1.5 mg of phospholipid P was added to each 100 ml of plasma each hour in normal birds and about 5 mg each hour in the birds treated with diethylstilbestrol. The total phospholipid of the plasma did not increase in the normal birds, so that about the same amount of phospholipid left the plasma in the same

time The total phospholipid of the plasma of the treated birds was increasing during the period of observation and less phospholipid left the plasma than entered A small amount of labeled phospholipid may have left the blood and returned to it during the period of observation but this amount would probably not greatly alter the figures obtained for the rate of synthesis by the liver The increased synthesis of phospholipid after administration of diethylstilbestrol to birds is probably greater than is indicated by the accumulation of phospholipid in the blood

The rate of disappearance of phospholipids from plasma has been studied in rabbits by Hahn and Hevesy (16), in dogs by Zilversmit and his associates (17) by means of the injection of plasma containing radioactive phospholipid, and in rats by means of the injection of emulsions of tissue phospholipids by Haven and Bale (18) In our small series of eight birds receiving radioactive phospholipid in donor plasma the rate of disappearance of phospholipid P from the plasma of the recipient varied from 1.3 to 3.06 mg for each 100 ml of plasma each hour as measured by the loss of radioactivity of the plasma phospholipid The change in the phospholipid as indicated by the change in the phospholipid content of the plasma varied from a loss of 2.4 mg of phospholipid P each hour to a gain of 0.85 mg The turnover rate of radioactively measured phospholipid appeared to be greater in those birds that had higher levels of phospholipid in the plasma, that is, in those that had received diethylstilbestrol The rate of turnover in the normal birds averaged 1.47 mg of phospholipid P for each 100 ml of plasma each hour as compared with 1.51 mg in the sixteen normal birds studied with radioactive sodium phosphate The average figure for the four treated birds was 2.76 mg each hour leaving the blood, as compared with 5.0 mg entering the blood in the sixteen treated birds studied with radioactive sodium phosphate

#### SUMMARY

When radioactive sodium phosphate was given intraperitoneally to cocks, radioactive phospholipid appeared in the plasma in 2 hours and increased at a uniform rate for 12 hours 6 hours after the administration of  $P^{32}$  the specific activity of the phospholipid of the plasma was similar in cocks which had received diethylstilbestrol and in untreated birds The concentration of phospholipid was greater in the plasma of the treated than of the untreated birds and the total radioactivity of the phospholipid correspondingly greater A greater amount of newly formed phospholipid had been added in the treated birds than in those not receiving diethylstilbestrol There was no significant difference between the treated and the untreated birds in the total amount and specific activity of the inorganic phosphate of the plasma and liver, the phospholipid content of the liver, or the glycerophosphate content of the liver In both groups the specific activity of the

inorganic phosphate of the liver was about the same as that of the plasma. There was a definite gradient in the specific activities of the inorganic phosphate, glycerophosphate, and phospholipid of the liver to the phospholipid of the plasma in the order given. From this standpoint glycerophosphate may be a precursor of phospholipid in the liver and the hepatic phospholipid be transferred to the blood.

Calculations based on the radioactivity of the phospholipid of the liver and the amount and radioactivity of the phospholipid of the plasma indicate that 1.51 mg of phospholipid P entered each 100 ml of plasma each hour in the untreated birds. Similar calculations showed an average of 5.0 mg entering the plasma each hour in birds which had received diethylstilbestrol.

Calculations based on the disappearance of radioactive phospholipid from the plasma of normal birds after intravenous injection of radioactive phospholipid in plasma indicated that 1.47 mg of phospholipid P left each 100 ml of plasma each hour. An average of 2.76 mg left the plasma each hour in four birds which had received diethylstilbestrol. Administration of diethylstilbestrol to birds appears to increase the rate of formation and the rate of turnover (utilization) of phospholipid.

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# THE THROMBOPLASTIC PROTEIN STRUCTURE, PROPERTIES, DISINTEGRATION\*

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The thromboplastic protein from beef lungs has formed the subject of a number of communications from this laboratory. These investigations included studies of the isolation of this agent by fractional salt precipitation (1) and by high speed sedimentation (2), of the composition of the lipids attached to the protein (3), of its electrophoretic behavior (2, 4), its particle weight (2), its immunological properties (1), and its reaction with heparin (5). A preliminary report on experiments aiming at the disintegration of this material by mild means was published recently (6).

The present article will contribute information on the following topics: the isolation of the thromboplastic protein from beef lung extracts by various centrifugal methods, the characterization of the nucleic acid and of the lipids contained in the complex, its disintegration by the action of ether, alcohol, and proteolytic enzymes, its activity as a clotting factor, and some of its enzymatic properties.

## EXPERIMENTAL

### *Isolation*

The isolation of the thromboplastic protein by the fractional ultracentrifugation of beef lung extracts has been described previously (2). This procedure leads, under proper conditions, to very active and homogeneous preparations (with respect to electrophoretic mobility and sedimentation velocity), but the isolation of monodisperse preparations is time consuming and suitable for the processing of only small lots. The sedimentation of lung extracts in a refrigerated International centrifuge equipped with a multispeed attachment or, if larger volumes are to be worked up, the combination of this procedure with a preliminary sedimentation in a Sharples supercentrifuge yields products that, although heterogeneous with respect to particle size, are equal in activity and electrophoretic homogeneity to the fractions obtained by the more laborious method.

The description of one experiment will be sufficient. Beef lungs, freshly

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obtained from the slaughterhouse, were ground and the material, which weighed 3870 gm, was extracted with 3900 cc of physiological saline for 1 hour at 4°. The mixture was pressed out through several layers of cotton gauze and the filtrate (3320 cc) passed rapidly through a turbine-driven Sharples laboratory supercentrifuge rotating at 50,000 R P M. The centrifuge was equipped with cooling coils (7) through which ice water circulated, whereby the contents of the clarifier bowl were maintained at a low temperature. The inside of the bowl was lined with a celluloid sheet. This operation, in which the widest delivery jet was employed, required about 8 minutes. The fluid remaining in the bowl was displaced by 200 cc of saline. The combined effluents were adjusted to a concentration of 1/15 M phosphate buffer by the addition of M buffer of pH 7. The sediment was suspended in the liquid remaining in the bowl and the mixture, similarly adjusted to pH 7, centrifuged in a refrigerated angle centrifuge at 4800 R P M (2700 g) for 30 minutes. The supernatant was united with the main solution which then was passed through the Sharples centrifuge, a fine delivery jet being used, at a very slow rate (16 cc per minute). 500 cc of the saline-phosphate buffer mixture were introduced for washing while the centrifuge was running. The copious pink sediment was removed from the celluloid liner and suspended in 200 cc of ice cold 0.1 M borate buffer of pH 8.5. The suspension was centrifuged for 90 minutes at 20,000 R P M (31,000 g) in a refrigerated International centrifuge equipped with a multispeed attachment. The suspension of the sediments in 100 cc of borate buffer was again subjected to centrifugation at the same speed. The pellets were suspended in 500 cc of borate buffer and the suspension was spun for 30 minutes at 8000 R P M (5000 g), in order to remove the more easily sedimentable material. This fraction was twice washed with borate buffer in the centrifuge at 5000 g, resuspended in borate buffer, and freed from a small amount of coarse material by centrifugation at 4000 R P M (1900 g) for 30 minutes. The supernatant was dialyzed for 24 hours against running tap water and for 96 hours against ice cold distilled water. The evaporation of the water from the frozen suspension in a vacuum yielded the material sedimentable at 8000 R P M (5000 g) as 7.02 gm of a slightly yellowish voluminous felt. This fraction contained N 5.8, P 2.0 per cent, N/P ratio 6.4. The reaction for acetal phosphatides (8) was positive.

The supernatant from this sedimentation which contained the thromboplastic protein was subjected to three more centrifugation cycles at 31,000 g and 5000 g alternately. The final solution, adjusted to an exact volume of 100 cc with borate buffer of pH 8.5, contained 1.75 mg of N and 0.38 mg of P per cc (N/P ratio 10.2). The total yield of the *thromboplastic protein* approximated 2.2 gm. Portions of this solution were used in experiments which will be described later, the remaining measured aliquot

was dialyzed for 48 hours each against running and ice-cold distilled water and was dried from the frozen state *in vacuo*, when Preparation 3 (Table I) was obtained as a white felt. This material was homogeneous in the electrophoresis cell, but polydisperse when examined in the analytical ultracentrifuge. Its electrophoretic mobility in borate buffer of pH 8.5 was  $-7.6 \times 10^{-5}$  sq cm per volt per second (descending and ascending boundaries).

Analytical data on other preparations of the thromboplastic protein, isolated by various centrifugal methods, will also be found in Table I. All substances gave a strongly positive reaction for acetal phosphatides (8).

TABLE I  
*Composition and Properties of Thromboplastic Protein Preparations*

Preparation No	Centrifugal method	Yield per kilo tissue	N	P	N/P	Thromboplastic activity†	Phosphatase activity	
							Phosphatase units per mg	Initial activity
		mg	per cent	per cent		$\gamma$		Assay
1	U	470	7.4	1.6	10.2			
2	M, U	440	7.8	1.5	11.5	0.003	1.6	2.8
3	S, M	535	7.6	1.6	10.5	0.008	1.6	4.5
4	M	480	7.8	1.6	10.8	0.003	2.2	3.8

\* U = air-driven vacuum ultracentrifuge, M = International multispeed centrifuge, S = Sharples laboratory supercentrifuge.

† Expressed as the smallest amount clotting 0.1 cc of rooster plasma (normal clotting time above 90 minutes) within 30 minutes.

### *Composition*

The thromboplastic protein preparations discussed here formed white or almost white voluminous fabrics which dispersed readily in slightly alkaline buffers to give markedly opalescent solutions, similar to the preparations described previously (2). All fractions gave a positive Molisch reaction. The fuchsin test for acetal phosphatides (8) was invariably positive, the diphenylamine reaction for desoxyribose nucleic acid (9) negative. A few, but not all, preparations exhibited a reddish brown color on the addition of iodine, resembling that given by glycogen.

**Lipids**<sup>1</sup>—For the extraction of the lipids, 398.0 mg of Preparation 3 (Table I) were suspended in 90 cc of a mixture of equal parts of absolute alcohol and ether, and the mixture was refluxed for 24 hours. The extraction residue, Fraction 3-P<sub>1</sub>, weighed 188.9 mg (47.5 per cent of the throm-

<sup>1</sup> The solvents used were purified and rectified by distillation. Ether was freed of peroxides. Whenever possible, the operations were carried out in a nitrogen atmosphere.



boplastic protein) and formed an almost white powder. It will be discussed in the next section.

The alcohol-ether extract was concentrated to dryness *in vacuo* and the solution of the residue in ether extracted twice with 10 per cent aqueous sodium chloride. The ethereal solution was dried with anhydrous sodium sulfate and the evaporation residue of the filtrate taken up in chloroform. The solution was filtered, evaporated to dryness *in vacuo*, and the residue dried to constant weight over  $P_2O_5$  *in vacuo*. The total lipids, Fraction 3-L, weighed 154.2 mg (38.8 per cent of the thromboplastic protein) and formed a light brown soft paste. The analytical composition of this fraction (in per cent) was found as follows: N 1.35, P 2.52, atomic N/P ratio 1.19, amino nitrogen (10) 0.17, amino nitrogen following hydrolysis with 5 N HCl for 18 hours at 100° 0.54, iodine value (11) 50.6, total cholesterol (12) 19.1 (there were practically no cholesterol esters), acetal phosphatides (13) 0.8 (calculated as palmitaldehyde).<sup>2</sup>

In another experiment, 214.0 mg of Preparation 1 (Table I) were extracted with 30 cc of absolute alcohol-ether (1/1) for 23 hours. The extraction residue, Fraction 1-Pr, weighed 106.5 mg (49.8 per cent of the thromboplastic protein). The evaporation residue of the extract was taken up in 2 cc of chloroform. The addition of 4 volumes of acetone to the solution, following centrifugation and concentration, precipitated the acetone-insoluble lipid fraction weighing 64.9 mg (30.3 per cent of the thromboplastic protein) and containing N 2.1, P 3.6 per cent. The acetone-soluble fraction weighed 35.0 mg (16.4 per cent of the thromboplastic protein). The separation had not been complete, as the acetone-soluble lipids contained an appreciable amount of P, *viz.* 0.84 per cent.

**Protein Moiety**—The residues from the alcohol-ether extraction of the thromboplastic protein gave the following analytical figures: Fraction 1-Pr, N 12.3, P 0.44, Fraction 3-Pr, N 13.4, P 0.38, amino sugar (14) 0.99 (calculated as glucosamine). Studies of the amino acid composition of these substances will be presented in a later report. Following hydrolysis, the presence of reducing substances in these preparations could be demonstrated. When, for instance, Fraction 3-Pr was subjected to hydrolysis at 100° with 1 N HCl for 1 hour, 13.3 per cent of reducing sugars (calculated as glucose) was found by the Hagedorn-Jensen method. This would correspond to a sugar content of 6.3 per cent in the thromboplastic protein. Preparation 3.

**Nucleic Acid**—A suspension of 53 mg of Preparation 1 (Table I) in 5 cc of 0.1 M acetate buffer of pH 4.9 was heated to boiling for 5 minutes.

<sup>2</sup> We are greatly indebted to Dr. W. M. Sperry for the cholesterol determinations, to Dr. H. Waelsch for the estimation of the acetal phosphatides, and to Mr. D. B. Sprinson for the determinations of amino nitrogen.

(compare (15)) The examination of the supernatant after centrifugation showed that 3.7 per cent of the total P of the thromboplastic protein had been liberated by this treatment. When examined in a Hilger spectrograph (at a concentration of 6.5  $\gamma$  of P per cc), the solution exhibited the ultraviolet absorption spectrum shown in Fig. 1, with a maximum at 2610 Å.

### Disintegration

*Freezing in Presence of Ether*—A preliminary account of some of these experiments was published recently (6). More complete data are assembled in Table II. All experiments were carried out in borate buffer of pH 7.7 (Experiments 1 and 5) or pH 8.5 (Experiments 2 to 4). Fresh

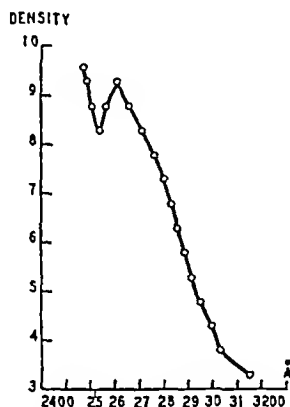


FIG. 1. Ultraviolet absorption spectrum of nucleic acid isolated from the thromboplastic protein (Preparation 1) by heat denaturation.

solutions of the thromboplastic protein preparations, as obtained following the final centrifugal purification, were employed in these operations. These samples had not undergone dialysis and drying.

In a typical experiment (Experiments 2, 3, Table II), the solution of 330 mg of Preparation 3 in 15 cc of borate buffer of pH 8.5 was mixed with 10 cc of ether, cooled for 4 minutes to  $-30^{\circ}$ , and permitted to thaw. The clear colorless ether layer was removed, 5 cc of fresh ether were added, and the freezing treatment was repeated six to eight times. The ether layer was replaced by fresh solvent each time. The mixture then was centrifuged, to effect a more complete separation of the layers, and the aqueous phase washed twice with ether. At this stage, most of the protein had collected in the interface, but after the removal of the remaining ether by careful evacuation, a homogeneous aqueous suspension resulted. It was

subjected to centrifugation at 4000 R P M (1900 *g*) for 30 minutes, when Fraction A (Table II) precipitated. Subsequent centrifugation at 20,000 R P M (31,000 *g*) for 90 to 120 minutes effected the sedimentation of small amounts of Fraction B, which appeared to be practically unaltered thromboplastic protein. The supernatant contained Fraction C, which was

TABLE II

*Disintegration of Thromboplastic Protein by Freezing in Presence of Ether*

Experiment No	Thromboplastic protein preparation No	Fraction No †	Proportion of starting material	λ	p	Thromboplastic activity‡	Phosphatase activity	
							Phosphatase units per mg	Initial activity
			per cent	per cent	per cent	γ		A <sub>100</sub>
1	2	2-A	50.0	8.1	1.3	0.001	1.6	3.0
		2-C	14.0	11.6	0.86	2	4.1	5.3
2	3	3-A	50.4	8.3	1.4	0.003	2.7	5.6
		3-B	7.5	8.1	1.5	0.008	2.9	4.4
		3-C	15.8	12.1	0.70	Inactive up to 6	2.0	4.5
						γ		
3	3	3-AA	48.3	8.0	1.2	0.003	1.5	3.4
		3-BB	11.8	8.4	1.2	0.008	4.8	8.0
		3-CC	15.9	12.3	0.49	Inactive up to 6	2.5	3.7
						γ		
4 (Control experiment)	3	C-3-B	98.1	7.2	1.6	0.003	0.9	1.8
		C-3-C	1.7			0.02	0.7	1.2
5	4	4-A	50.8	8.1	1.4	0.008	1.2	1.8
		4-C	16.7			2	1.7	1.7

\* The numbers of the preparations refer to Table I

† The disintegration products are designated by the numeral corresponding to the preparation used, followed by letters defining the centrifugal characteristics of the fraction: A, almost complete sedimentation at 1900 *g*; B, no sedimentation at 1900 *g*; C, complete sedimentation at 31,000 *g*; D, no sedimentation at 31,000 *g*. Duplication of the letters denotes the repetition of an experiment. The control experiment in which ether was omitted is designated by C preceding the number.

‡ Expressed as the smallest amount clotting 0.1 cc of rooster plasma (normal clotting time above 90 minutes) within 30 minutes.

found to consist of a mixture of non-sedimentable proteins. In one case, *viz* Fraction 3-C (Table II), an electrophoretic study was carried out which revealed the presence of three components with the following mobilities and relative proportions (borate buffer of pH 8.5, descending boundaries): I, -3.3 (25 per cent), II, -6.6 (46 per cent), III, -8.1 (29 per cent)  $\times 10^{-5}$  sq cm per volt per second. It will be remembered that the

thromboplastic protein Preparation 3, which formed the starting material, was homogeneous electrophoretically and had a mobility of  $-7.6$ . The A fractions gave a positive, the C fractions a negative Feulgen test for acetal phosphatides (8).

Control experiments, carried out simultaneously, in which the ether was omitted (*e.g.* Experiment 4, Table II), failed to show any gross changes in the protein due to the freezing and thawing, no appreciable disruption or aggregation was observed. The sedimentation of the protein (Fraction C-3-B), negligible at 1900 *g*, became almost complete at 31,000 *g*. The supernatant contained only traces of non-sedimentable protein (Fraction C-3-C).

All fractions were finally suspended in buffer, dialyzed against running and ice-cold distilled water, and recovered by the evaporation of the water in the frozen state in a vacuum.

For the recovery of the liberated lipids, the ethereal extracts from Experiments 2 and 3 (Table II) were combined, extracted several times with 10 per cent aqueous sodium chloride, and dried with anhydrous sodium sulfate. The lipid mixture was taken up in chloroform and then again in ether and the solutions were clarified each time. The lipid preparation weighed 120.8 mg (18.3 per cent of the thromboplastic protein) and contained N 0.85, P 1.5. It gave a strong reaction for acetal phosphatides (8). The comparison of yield and composition of this fraction with those of the total lipid preparation obtained by extraction with hot alcohol-ether, discussed in a preceding section, shows that the lipids removed by freezing in the presence of ether amounted to about one-third of the total lipids present. Their composition was significantly different, inasmuch as a relatively larger proportion of non-phospholipid material appeared to be removed by the freezing process.

The treatment of the disintegration products with hot alcohol and ether removed additional lipid material, as exemplified by the following analytical figures: Fraction 3-AA (extracted), N 12.8, P 0.46, Fraction 3-CC (extracted), N 14.3, P 0.22.

*Action of Alcohol*—A solution of 110 mg of thromboplastic protein (Preparation 3) in 5 cc of borate buffer of pH 8.5 was shaken with 15 cc of absolute alcohol-ether (1:9) for 1 minute in the cold. Centrifugation of the mixture at 4000 R P M (1900 *g*) for 30 minutes effected the separation of an only slightly turbid aqueous phase, a thick jelly which collected at the interface, and a yellow ether layer. The ether layer was removed and the aqueous layer, together with the jelly, was repeatedly washed with ether in the centrifuge. The removal of the remaining ether by careful evacuation was followed by centrifugation at 20,000 R P M (31,000 *g*) for 90 minutes, dialysis of the suspended sediment and of the supernatant, and vacuum concentration of the frozen solutions.

The sedimentable material, Fraction 3-D, formed 76.4 mg (69.5 per cent of the thromboplastic protein) of a slightly yellowish felt, the non-sedimentable portion from the supernatant, Fraction 3-E, consisted of a voluminous fluff, weighing 20.0 mg (18.2 per cent). The lipid fraction, isolated from the ether extract in the usual manner, weighed 15.7 mg (14.3 per cent) and formed a brown paste which gave a positive reaction for acetal phosphatides. Fraction 3-D likewise gave a positive Feulgen reaction, whereas Fraction 3-E gave none. Additional evidence of the presence of lipids in Fraction 3-D was afforded by the effect of hot alcohol-ether on this material. The resulting Fraction 3-D (extracted) had a significantly changed composition. Analytical data on these fractions are compared in Table III.

*Action of Proteolytic Enzymes*—In one set of experiments, the action of crystalline trypsin and chymotrypsin<sup>3</sup> on the thromboplastic protein

TABLE III

*Disintegration of Thromboplastic Protein (Preparation 3) by Means of Alcohol*

Fraction No	Proportion of starting material	N	P	Thromboplastic activity	Phosphatase activity	
					Phosphatase units per mg	Initial activity
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	$\gamma$		$A_{100}$
3-D	69.5	8.0	1.4	0.2	1.8	2.5
3-D (Extracted)		12.8	0.41			
3-E	18.2	10.4	0.63	6	0	0
Lipids	14.3		0.73			

\* Expressed as the smallest amount clotting 0.1 cc of rooster plasma (normal clotting time above 90 minutes) within 30 minutes.

Preparation 1 (Table I) and on the corresponding extraction residue (Fraction 1-Pr), resulting from the removal of lipids by means of hot alcohol-ether, was examined. The suspensions of the dried substances in 0.1 M borate buffer of pH 7.8 contained per cc 0.73 mg of Preparation 1 or 1.3 mg of Fraction 1-Pr and 20  $\gamma$  of trypsin or chymotrypsin respectively. The mixtures were incubated at 37° and the disaggregation of the suspended particles was followed turbidimetrically in a Klett-Summerson photoelectric colorimeter. The turbidity curves obtained with trypsin<sup>4</sup> are reproduced in Fig. 2.<sup>5</sup> An experiment with a freshly prepared thromboplastic protein preparation (similar to Preparation 3, Table I), which had

\* These enzyme preparations were kindly placed at our disposal by Dr. M. Kunitz of the Rockefeller Institute, Princeton.

<sup>4</sup> The curves observed with chymotrypsin were almost completely identical.

<sup>5</sup> The instrument used gave a reading of 118 for No. 2 and of 234 for No. 4 of the nephelometric barium sulfate scale of McFarland (16).

not undergone drying, is likewise included as Curves I, *a*, *b* (3.2 mg of protein, 20  $\gamma$  of trypsin per cc of borate buffer of pH 7.8). It will be seen that the decrease in turbidity was much greater with the lipid-free Frac-

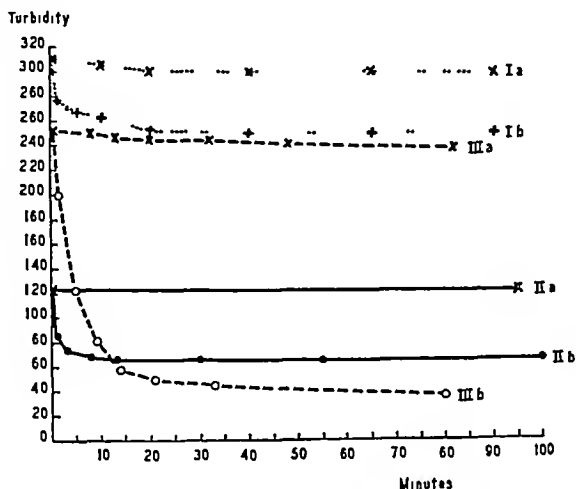


FIG 2 Influence of crystalline trypsin on turbidity of thromboplastic protein preparations. Curve I, 0.3 per cent solution of thromboplastic protein, Curve II, 0.07 per cent suspension of Preparation 1, Curve III, 0.13 per cent suspension of lipid free Fraction 1-Pr. Control experiments are shown as *a*, tryptic digestion experiments as *b*. Borate buffer, pH 7.8, 37°

TABLE IV

*Action of Crystalline Trypsin and Chymotrypsin on Lipid-Free Thromboplastic Protein (Fraction 1-Pr)*

	Substrate before digestion	Trypsin				Chymotrypsin		
		Before dialysis	After dialysis	Dialyzable portion		Before dialysis	After dialysis	Dialyzable portion
	$\gamma$ per cc	$\gamma$ per cc	$\gamma$ per cc	per cent		$\gamma$ per cc	$\gamma$ per cc	per cent
N	161	133	84	37		137	95	31
P	5.7	4.5	3.6	20		4.5	4.1	9
Carbohydrates (as glucose)		85	76	11		85	81	5

tion 1-Pr (Curves III, *a*, *b*) than with the intact thromboplastic protein fractions (Curves I, *a*, *b* and II, *a*, *b*)

The mixtures containing the lipid-free Fraction 1-Pr and trypsin or chymotrypsin were, after 4 hours at 37°, centrifuged at 4000 R.P.M. and the supernatants, following the removal of aliquots for analysis, dialyzed overnight. The results are summarized in Table IV. The carbohydrate

determinations were carried out by the orcinol method (17, 18). The values in Table IV, corrected for changes in concentration during dialysis, show that a large portion of the suspended substrate was brought into solution by either enzyme. It is worthy of note that a considerable proportion of the nitrogen contained in the protein was converted into a dialyzable form, whereas most of the phosphorus and carbohydrates remained undialyzable.

### *Thromboplastic Activity*

**Clotting of Plasma**—The assays were carried out with rooster plasma in the usual arrangement (2, 19). The experiments were performed at 30.6° by mixing 0.1 cc of fresh plasma with 0.03 cc of the solution of the protein in borate buffer of pH 8.5. For purposes of comparison, the activities are expressed as the smallest amount clotting 0.1 cc of plasma within 30 minutes. The results obtained with various preparations of the thromboplastic protein will be found in Table I. The activities of the disintegration products obtained by freezing in the presence of ether and by the action of alcohol are summarized in Tables II and III respectively. It may be of interest to present here the assay protocol for one highly active fraction, *viz.* Fraction 3-A (Table II, Experiment 2), in order to illustrate the truly remarkable potency of these preparations which in this case permitted the demonstration of  $3 \times 10^{-10}$  gm

	Thromboplastic protein in experiment							
	0.22 $\gamma$	0.074 $\gamma$	0.024 $\gamma$	0.008 $\gamma$	0.0027 $\gamma$	0.0009 $\gamma$	0.0003 $\gamma$	0 $\gamma$
Clotting time, min	3	5	9	15	21	34	47	>90

The extremely heavy material which, as described in the first section of the experimental part, is removed from the solution of the thromboplastic protein by sedimentation at 8000 R.P.M. (5000 *g*) possesses little thromboplastic activity. The smallest amount of this fraction that clotted 0.1 cc of plasma within 30 minutes was found to be 2  $\gamma$ .

**Action on Prothrombin**—The preparations of the thromboplastic protein were free of thrombin. The conversion of prothrombin to thrombin under the influence of the purified thromboplastic protein was followed in a number of instances. The purified prothrombin used, for which we are highly indebted to Dr. W. H. Seegers, Parke, Davis and Company, Detroit, had an activity of about 2000 units per mg of nitrogen (compare (20, 21)). Human fibrinogen was employed in a technique essentially similar to that of previous experiments (22). To 0.1 cc of a 0.1 per cent solution of prothrombin in physiological saline, 0.1 cc of the thromboplas-

tic protein (Table I, Preparation 3) dilution in saline containing 0.15 per cent calcium nitrate were added. After 20 minutes at room temperature (30°), 0.03 cc of the mixture was placed in a small test-tube and 0.2 cc of a 1.4 per cent fibrinogen solution in phosphate buffer of pH 7 was added. In the following clotting time determinations, which represent the average of a number of tests carried out at 30°, each tube contained the equivalent of 15  $\gamma$  of prothrombin, 2.8 mg of fibrinogen, and the indicated amounts of the thromboplastic protein.

	Thromboplastic protein in experiment							
	3 $\gamma$	1 $\gamma$	0.33 $\gamma$	0.11 $\gamma$	0.037 $\gamma$	0.012 $\gamma$	0.004 $\gamma$	0 $\gamma$
Clotting time, sec	14	15	26	33	57	113	148	>7200

In other experiments, the heat stability of the thromboplastic protein was studied. The experimental arrangement was similar to the one men-

TABLE V  
*Stability of Thromboplastic Protein*

	Thromboplastic protein in experiment					
	3 $\gamma$	1 $\gamma$	0.33 $\gamma$	0.11 $\gamma$	0.037 $\gamma$	0 $\gamma$
	sec	sec	sec	sec	sec	sec
Unheated	9	11	13	18	27	>7200
Heated	10	10	10	15	30	>7200

tioned above, with the exception of the temperature (25°) and the fibrinogen concentration (0.7 per cent). A portion of the 0.02 per cent solution of the thromboplastic protein (Preparation 3) was heated to 80° for 20 minutes. Dilutions of both the unheated and the heated solutions were then incubated with prothrombin and tested as described before. (See Table V.)

The stability of the thromboplastic protein was much less marked when more dilute solutions of the substance (20  $\gamma$  per cc and less) were exposed to heat. In this case, complete or partial inactivation appeared to take place.

#### *Phosphatase Activity*

The phosphatase activity of the thromboplastic protein preparations and their disintegration products is summarized in Tables I to III. The determinations were carried out in the presence of magnesium ions with sodium  $\beta$ -glycerophosphate as the substrate. For the experimental arrangement and the definition of the units employed, reference should be



made to a previous publication (2) The determination of the phosphatase units is based on the work of Albers (23), the computation of the initial activity on studies of Bodansky (24)

The fraction sedimentable at 8000 R P M (5000 *g*) contained 1.4 phosphatase units per mg and had an initial activity  $A_{100} = 3.0$

The incubation of thromboplastic protein preparations with crystalline trypsin or chymotrypsin in the experimental arrangement described before was found to be without influence on the phosphatase activity

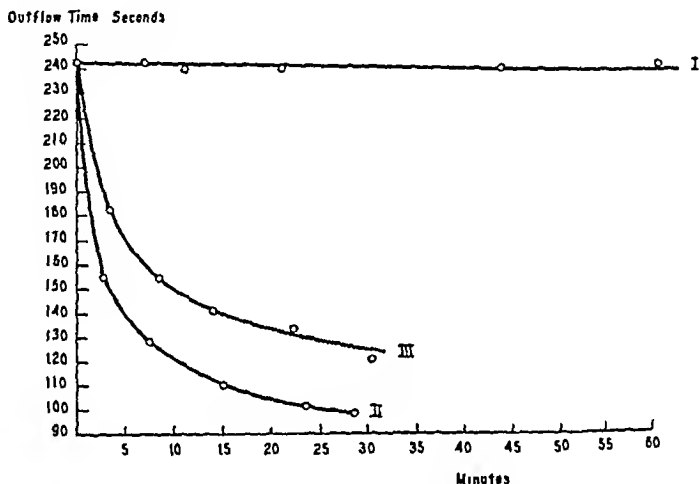


FIG 3 Effect on viscosity of gelatin Curve I, thromboplastic protein Preparation 1, 5.0 mg in 5 cc of gelatin, Curve II, crystalline trypsin, 55  $\gamma$  in 5 cc of gelatin, Curve III, thromboplastic protein Preparation 1 (1.7 mg) and crystalline trypsin (55  $\gamma$ ) in 5 cc of gelatin Phosphate buffer, pH 7.4, 34°

### *Examination for Proteolytic Action*

A preceding article (2) included a report on attempts to test for the presence of a trypsin-like activity in the thromboplastic protein by means of a titrimetric procedure with benzoylargininamide as substrate The results were, however, inconclusive, since the slow decomposition of the protein itself prevented accurate determinations For this reason, the experiments were repeated with a method that did not rely on changes in acidity The procedure adopted finally was that of Northrop and Hussey (25) which employs the decrease in viscosity of a gelatin solution as the criterion of proteolytic action A number of preparations (Preparations 1 and 3 in Table I, Fractions 3-AA and 3-CC in Table III) was tested in concentrations from 1 to 5 mg per 5 cc of gelatin solution The arrangement was

similar to the one described by Northrop and Hussey (25) The experiments were carried out in phosphate buffer of pH 7.4 at 34° by means of an Ostwald viscosimeter (outflow time 68.7 seconds for water at 34°)

None of the substances tested had even the slightest influence on the viscosity of gelatin An example will be found as Curve I in Fig. 3 The adequacy of the experimental procedure was proved by the viscosity decrease produced by crystalline trypsin, chymotrypsin, and commercial trypsin (Fairchild) The effect of crystalline trypsin is illustrated by Curve II in Fig. 3 Peculiar results were obtained when mixtures of thromboplastic protein and trypsin were examined the tryptic action was invariably found to be inhibited to a certain extent An example is shown as Curve III in Fig. 3 The thromboplastic protein was, however, without effect on the action of chymotrypsin

#### DISCUSSION

The experiments here presented demonstrate the possibility of isolating large quantities of the thromboplastic protein from beef lung extracts by means of a cooled Sharples supercentrifuge This was to be expected, in view of the very high particle weight of the substance (2), since a similar instrument has been used for the isolation of the tobacco mosaic virus (7) The further purification of the thromboplastic protein preparations thus obtained requires the employment of a refrigerated high speed centrifuge

The protein preparations which, it should be remembered, are isolated by very mild methods and are not in contact with organic solvents in the course of their preparation lose about one-half of their weight by exhaustive extraction with hot alcohol-ether Between 40 and 45 per cent of the material may be recovered as purified lipids The composition of the total lipid fraction could, on the basis of the analytical figures, be tentatively expressed as follows (in per cent of total lipids) cholesterol 19 (almost exclusively in the free state), fat 18, phospholipids 63 ("lecithin" 26, "cephalin" 25, "sphingomyelin" 12) The acetal phosphatide content of the lipid fraction may be estimated as about 1.5 per cent The lipids attached to the thromboplastic protein preparation obtained by fractional salt precipitation (1), which perhaps was contaminated with the coarsely particulate fraction sedimentable at 8000 R P M, formed the subject of a previous more detailed study (3)

The residue remaining from the extraction of the lipid portion consists in the main of proteins, some carbohydrates, and a nucleic acid of the ribose nucleic acid type If the phosphorus content of this material (0.38 per cent) is assumed to be entirely due to nucleic acid, the presence of about 18 per cent of ribose nucleic acid in the intact thromboplastic protein would be indicated The electrophoretic homogeneity of the complex

speaks against the assumption that nucleic acid is present as an impurity. It appears to be attached to the protein by fairly labile bonds. In preparations that had undergone more extensive chemical manipulation than the ones discussed here, the electrophoretic examination did reveal the existence of a small amount of a separate faster moving compound which carried most of the non-lipid phosphorus and probably consisted of nucleic acid liberated in the course of the isolation of the complex (4). The lability to heat of the ribose nucleic acid-protein bond was demonstrated in recent work on the tobacco mosaic virus (15) and has also been observed in the present studies of the thromboplastic protein.

The question may be raised, whether one is entitled to regard the thromboplastic protein and similar lipoproteins as compounds between proteins and lipids or whether the latter occur in purely physical association. The appearance of complexity in structure of the macromolecular thromboplastic protein, which this paper serves to emphasize, is mainly a reflection of our lack of understanding of the modes of linkage prevailing between its various constituents. To give even a partial catalogue of our ignorance on this subject would require more space than can be afforded here, and reference should be made to a recent review article on lipoproteins (26). But there are certain features that serve to distinguish genuine lipid-protein complexes from simple mixtures or loose adsorption systems. In general, the term lipoprotein may be said to designate a group of compounds whose properties, e.g. biological reactivity, solubility, physical characteristics, etc., differ from those of the sum of their components. In the case of the thromboplastic protein, for instance, it would be hard to understand how the large amounts of water-insoluble lipids and steroids present could escape separation from the protein portion, in the course of the elaborate centrifugal fractionation procedure, unless they are in chemical combination with the protein. The behavior of these complexes towards organic solvents (26) may be mentioned as another remarkable characteristic.

The thromboplastic protein of beef lungs resembles in certain respects, e.g. the presence of ribose nucleic acid and of acetal phosphatides, the submicroscopic particles isolated from a number of tissues (compare (27, 28)). Its nitrogen content appears to be considerably lower than that of the liver particles for which a figure of 9.1 per cent has been reported (27). Whether the similarity between these substances, which are probably of cytoplasmic origin, is more than superficial will have to be decided by detailed chemical and immunological studies. Data on some immunological properties of particulate tissue proteins, including lung particles, have been reported (29, 30).

The assays of the thromboplastic potency of the preparations reported

in Tables I to III and in the section on the activation of purified prothrombin revealed a very high degree of activity. In some cases, amounts as small as 0.0003  $\gamma$  of the thromboplastic protein still exhibited a noticeable potency. This agent appears to be comparatively stable to heat. It is possible that the high lipid content has a protective function in this regard. The heat stability of thromboplastically active crude tissue extracts was remarked upon more than 30 years ago ((31) p. 529). The old distinction between thermostable thromboplastic agents (lipid factor) and thermolabile ones (protein factor) would, therefore, seem to lose some of its sharpness.

The experiments on the disintegration of the thromboplastic protein by freezing in the presence of ether are based on an observation of McFarlane (32). This author found that a large proportion of the serum lipids, ordinarily not extractable with ether, was transferred into the ether phase when ether-containing serum was frozen to a temperature below  $-25^{\circ}$  and then allowed to thaw. By a similar treatment, the thromboplastic protein has been found to break into four principal fractions. (1) About one-half of the material is aggregated to form coarse particles sedimentable at 4000 R.P.M. (Fractions A, Table II). The N/P ratio and the phosphatase activity of this fraction are changed only slightly, the thromboplastic potency is somewhat higher than that of the starting material. (2) A small fraction (Fractions B, Table II) shows the centrifugal characteristics and the thromboplastic activity of the unaltered starting material, but has, in general, a higher phosphatase potency. (3) The supernatant from these two fractions contains a considerable proportion (14 to 17 per cent) of a mixture of non-sedimentable proteins (Fractions C, Table II). This fraction, which in one experiment could be shown to consist of three distinct electrophoretic components, is devoid of thromboplastic activity, but is quite active as phosphatase. (4) A lipid fraction amounting to about 18 per cent of the starting material, *i.e.* roughly one-third of the total lipids of the thromboplastic protein, is recovered from the ether phase. Control experiments, in which the freezing was carried out in the absence of ether, failed to reveal any evidence of a similar disintegration (compare Experiment 4, Table II).

The disintegration experiments with ether containing 10 per cent of alcohol (Table III) do not require any extended comment. They showed that even brief contact with alcohol sufficed to bring about an almost complete destruction of the thromboplastic potency and a far reaching interference with the phosphatase activity. In this case, too, the disruption of the thromboplastic protein was accompanied by the detachment of a non-sedimentable protein fraction and of lipids.

The experiments on the effect of trypsin and chymotrypsin demonstrate

that a portion of the thromboplastic protein can be digested by proteolytic enzymes. It should be of interest, as part of a general study of the physiological function of lipoproteins, to investigate whether the lipids contained in the intact complex are able to protect the protein moiety from the enzymatic attack.

The phosphatase activity of the thromboplastic protein (2) and of its disintegration products was followed, not because of any possible connection between phosphatase action and the activation of prothrombin (there probably is none) but as an indicator of the adequacy of the methods used for the splitting of the complex. It is known that the phosphatase present in kidney particles may be released in a non-sedimentable form by autolysis (33). Similarly, tryptic digestion has been used to effect the liberation of intestinal phosphatase from tissue particles (34). The experiments, mentioned in the present article, on the digestion of the thromboplastic protein by proteolytic enzymes in the course of which the phosphatase activity remained unchanged, are reminiscent of these findings.

The examination of the thromboplastic protein for a trypsin-like action, which had previously led to inconclusive results (2), was resumed in the present studies by a different technique. No indication of any effect on gelatin, which served as the substrate, could be found. The thromboplastic protein had, in fact, an inhibiting action on crystalline trypsin, perhaps because of the competition between two substrates (gelatin and thromboplastic protein) for the enzyme.

The findings regarding the disruption of the thromboplastic protein by freezing in the presence of ether may perhaps throw some light on the structure of this lipoprotein. X-ray studies of similar substances have shown that these complexes occur as thin protein layers inserted between bimolecular lipid leaflets (35). This view permits the assumption that these units could arrange in a regular manner to form large complexes whose size would perhaps be limited by the intracellular spaces in which their formation takes place. The importance of the lipids in maintaining an automatic uniformity of particle size and electrophoretic mobility could thus be understood. Once the protective water barrier is frozen away, the uniformity of the ostensibly homogeneous complex disappears owing to the removal of lipids by the ether, and separation into discrete components takes place.

The authors are highly indebted to Dr. D. H. Moore for the electrophoresis experiments. They are very grateful to Miss Helen Fabricant for technical assistance and for help with some of the analytical determinations.

## SUMMARY

This paper describes continued work on the thromboplastic protein of beef lungs, an agent representative of the tissue factors which initiate the blood-clotting process by their action on prothrombin. The isolation of this high molecular lipoprotein by various centrifugal means is discussed. Studies of the composition of the lipid fraction and indications of the presence of ribose nucleic acid, both in combination with a protein component, are presented.

This substance is shown to be disintegrated into a number of fractions by freezing in the presence of ether. The disruptive effect of alcohol and of proteolytic enzymes has likewise been studied. The activities of the products are discussed with respect to thromboplastic and phosphatase potencies. A remarkably high thromboplastic activity (tested with both plasma and prothrombin) has been found in some of the fractions.

No indication of a trypsin-like activity of the thromboplastic protein could be found when the decrease in viscosity of a gelatin solution was used as the criterion of enzyme action.

The article concludes with a discussion of the possible function of the lipids in maintaining the architecture of the thromboplastic protein.

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# CHOLESTEROL INJURY IN THE GUINEA PIG

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When excessive amounts of cholesterol are deposited in a tissue, the nature and permanence of the resulting injury may logically be expected to depend on the rate of its removal and on the mechanism involved. Previous work in this laboratory (1) suggested that it might be relatively easy to follow the process of clearing out cholesterol deposits from the livers of guinea pigs which had been fed cholesterol. This was because the severity of the anemia which resulted from cholesterol feeding in this species had been found to be related to the amount of cholesterol in the liver. Other characteristic responses to cholesterol feeding manifested by the guinea pig also promised interesting possibilities in a study of its recovery from the resultant injury. These were, notably, increases in free as well as ester cholesterol in the liver and blood, with penetration of extra cholesterol to all parts of the body, gross enlargement of the spleen, hyperplasia of the bone marrow, and, sometimes, formation of gallstones.

## *Experimental Procedure and Results*

The plan for the study here reported called for alternate periods of cholesterol feeding and withdrawal, with observation of the body weight, the blood picture, and the chemical and structural characteristics of various tissues at the different stages of injury and recovery. Because considerable time was required for the determinations and because this study was carried on parallel with other work, only a few animals were under observation at any one date, and the study extended over several years.

Young guinea pigs were placed on basal diets of a type that had proved adequate for normal growth in control animals. For the periods of cholesterol injury, 1 per cent cholesterol was dissolved in the fat and incorporated into the diet. For the periods of recovery the animals were returned to the basal diets made up without cholesterol. Table I gives the composition of the two diets used, together with the composition of the vitamin supplements.

The guinea pigs were kept in individual wire cages. They had access at all times to the basal diet and to clean water from glass bottles. Vitamin supplements were given by pipette three times weekly, just after the animals were weighed.



Erythrocyte counts were made on blood from ear veins, as were reticulocyte counts and hemoglobin determinations. At autopsy, total weights of liver and spleen were taken. Samples of tissues were prepared for moisture and lipid determinations. The technique used in the lipid analyses has already been described (3). Frozen sections, fixed in 10 per cent formalin, were used for study of fat distribution, and paraffin sections, stained with hematoxylin and eosin, for study of tissue structure. Detail of the histological findings is not included in this paper.

TABLE I  
*Basal Diets Fed ad Libitum\**

	Diet A		Diet B	
	Control	Cholesterol	Control	Cholesterol
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Casein, commercial	20 0	20 0	20 0	20 0
Fat (Primex)	15 0	15 0	12 5	12 5
Wheat bran	5 0	5 0	5 0	5 0
Dried brewers' yeast	10 0	10 0	10 0	10 0
Salts (Hubbel, Mendel, Wakeman (2))	2 5	2 5	2 5	2 5
Agar	2 5	2 5	2 5	2 5
Corn-starch	45 0	44 0	47 5	46 5
Cholesterol	0 0	1 0	0 0	1 0

\* 10 gm of each diet were shown by micro assay to furnish approximately 90  $\gamma$  of thiamine, 90 to 120  $\gamma$  of riboflavin, 500  $\gamma$  of nicotinic acid, and 140  $\gamma$  of pantothenic acid. Vitamin supplements were given by pipette three times weekly in addition to the basal diet. All animals, regardless of notation, were given in terms of daily dose equivalents, 25  $\gamma$  of thiamine, 3 cc of orange juice containing 2.5 mg of added ascorbic acid, 2 drops of a cottonseed oil solution containing 75 i.u. of vitamin A as carotene, and 25 i.u. of vitamin D as irradiated ergosterol, and 1 drop of wheat germ oil. In addition to the above, certain groups were given the following "c" 0.25 gm of dried grass tips (Cerophyl), "r" 25  $\gamma$  of riboflavin, and "p" 125  $\gamma$  of calcium pantothenate. In Table III the diets are indicated by capital letters and the extra supplements by lower-case letters. For example, Diet A<sub>c</sub> means basal Diet A with extra supplement "c."

No samples were taken from animals found dead, and complete tissue examination was, for lack of time, limited to representative animals from each group as determined by weight and growth, red cell count, and liver weights. Tables II and III represent, therefore, only a fraction of the data from forty-eight experimental and thirty-two control animals used in the study.

Figs 1 and 2 show the changes in body weight, together with erythrocyte and reticulocyte counts, in two typical animals during development of and recovery from cholesterol anemia. Table II shows distribution of chole-

terol in corpuscles and plasma of typical animals Table III summarizes data on the relation of size of liver, size of spleen, red blood cell count, and liver lipids for typical animals on which complete series of observations were made Changes in chemical composition of various organs, together with a general indication of the nature of the observed structural changes, are given below

*Livers*—Development of a fatty liver with a high cholesterol ester content was a relatively prompt response to cholesterol feeding in the guinea pigs In contrast, removal of the cholesterol ester deposits was extremely slow (Table III) In only one of the three animals for which complete liver lipid analyses were made at the time the red blood cell count had returned to normal had the liver cholesterol ester also returned within normal range This animal had been on a nearly cholesterol-free basal diet for 147 days after its period of cholesterol feeding, and even in this liver the lecithin level (2.8 per cent) was well under the average for our control guinea pigs

TABLE II  
*Distribution of Lipids in Blood of Guinea Pigs*

Average of 6 guinea pigs		Total cholesterol	Free cholesterol	Lecithin	Ratio of lecithin to free cholesterol
		mg per cent	mg per cent	mg per cent	
Corpuscles	Cholesterol-fed	400	337	471	1.39
	Controls	266	199	328	1.65
Plasma	Cholesterol-fed	236	137	109	0.79
	Controls	87	51	49	0.96

In general, the lecithin content of the liver rose as cholesterol content fell, and liver lecithin values for control animals, even on the basis of moist weight, were 50 per cent higher than those for animals on cholesterol diet

Structurally, the livers of animals placed on the cholesterol diet first showed a diffuse invasion of hepatic cells by small fat droplets Then the lipid tended to accumulate in larger aggregates, and there was evidence not only of distortion, but also of breakdown of hepatic cell structure Animals killed some time after removal of cholesterol from the diet, but while the livers were still markedly fatty, often showed groups of phagocytic cells surrounding the larger lipid droplets During the course of lipid removal there was no entirely uniform pattern of distribution of droplets left behind The comparatively minor accumulations of what may be regarded as scar tissue in livers in which cholesterol content had returned to normal may perhaps be accounted for by the fact that a guinea pig seldom survived long if it had extensive areas of necrosis in a fatty liver

Judging from the livers of animals killed after a second feeding period

TABLE III  
Summary of Data on Typical Guinea Pigs

Guinea pig No *	Diet†	Age started	Days fed diet	Low r b c	Days off diet	R b c at death	Body weight gm	Liver weight gm	Spleen weight gm	Gall stones	Liver lipids per cent moist weight				
											Fatty acids	Total cholesterol	Free cholesterol		
Controls, basal diet, no cholesterol															
Average (5)	A	Un-known	65	million	0	million	gm	gm	gm	—	5 1	0 47	0 34	3 4	
Range			60-70		0	5 3-6 7	402-480	14	0-19	40	2 6-9 0	0 30-0 76	0 28-0 43	1-3 8	
Average (4)	Bcr	22	82		0	5 6	703	24 8	0 96	—	4 7	0 61	0 29	3 1	
Range		22-22	77-84		0	5 5-5 7	600-800	23	7-27	0 74-1 1	—	3 0-7 8	0 51-0 71	0 27-0 33	2 8-3 4
Average (4)	Bcrp	47	120		0	5 8	680	24 0	0 83	—	4 8	0 63	0 29	3 3	
Range		40-49	91-184		0	5 5-6 2	600-760	22	9-25	30	3 5-7 3	0 33-0 83	0 22-0 35	3 0-3 7	
Animals killed while on diets containing cholesterol															
412 ♂	A	Un-known	36	3 5	0	3 5	278	32 6	4 1	—	17 1	3 3	0 61	1 7	
413 ♀	"	"	43	2 1	0	2 1	314	40 7	9 4	—	20 2	3 7	0 69	1 9	
591 ♀	Bcrp	71	40	4 1	0	4 1	320	25 9	5 0	—	10 3	3 3	0 60	2 3	
594 ♂	"	45	85	3 0	0	3 0	560	54 8	6 8	—	24 1	5 7	0 57	1 8	
595 ♀	Bcr	71	76	1 6	0	1 6	560	62 0	12 2	+	17 0	4 0	0 47	2 0	
599 ♂	"	37	63	3 1	0	3 1	360	41 0	2 5	+	19 8	4 3	0 76	2 1	
Animals killed after various periods off cholesterol, little evidence of recovery from anemia															
438 ♀	Ac	60	62	1 5	4	1 5	416	42 1	5 3	—	18 3	4 7	7 1	2 0	
583 ♀	Bcrp	60	49	2 3	15	3 3	430	48 5	6 2	+	13 6	4 1	0 52	2 0	
589 ♀	Bcr	65	42	2 0	36	3 3	470	46 4	18 4	+	21 0	3 8	0 56	2 1	
441 ♂	Ac	57	60	2 5	98	3 2	585	29 8	18 9	+	11 1	3 6	0 35	2 4	
435 ♂	"	62	60	2 0	118	3 6	700	51 7	9 2	+	11 1	3 7	0 41	2 8	

Animals showing some evidence of recovery from anemia

569 ♂	Bepr	63	50	1 9	75	5 1	710	42 6	2 3	+	11 7	2 3	0 49	2 7
165 ♀	A		42	2 0	88	4 4	478	24 4	6 8	-	8 2	3 4	0 66	2 7
566 ♂	Be	66	66	2 2	146	5 6	810	42 0	4 4	+	13 4	2 1	0 30	2 6
434 ♂†	Ac	62	75 + 22	2 2	89 + 8	4 1	800	61 4	16 9	-	21 0	4 7	0 64	2 2
436 ♀	"	63	47	2 7	147	5 5	850	35 0	6 2	+	4 1	0 58	0 30	2 8
582 ♀	Be	63	35	1 9	219	3 9		37 3	4 9					
593 ♀§	"	71	43 + 6	1 8	6 + 23	1 5	478	49 9	8 0	+	18 2	3 2	0 45	1 5

\* The figures in parentheses indicate the number included in the average

† See the foot-note to Table I

‡ Fed cholesterol 75 days, returned to basal diet for 89 days, when the red blood cells reached 5.6 million, then returned to cholesterol for 22 days, when the red blood cells were 2.4 million. The animal was again returned to basal diet and killed after 8 days.

§ Fed cholesterol 43 days, returned to basal diet for 6 days, when the red blood cells were 4.9 million, return to cholesterol lowered the count to 1.5 million in 6 days. The animal was then returned to basal diet for 23 days and sacrificed.

with cholesterol, their rapid breakdown may have been due to poor recovery from previous hepatic cell injury rather than to blocking of circulation by scar tissue. Lack of uniformity of cell pattern within individual lobes was, however, an outstanding feature of these particular liver slides.

Efforts to determine the localization of the cholesterol and to distinguish it from other liver lipids by means of the petrographic microscope were unsuccessful. This may have been because the cholesterol was in solution in fat and the solution was not birefringent. The Liebermann-Burchard reaction applied to dried gelatin mounts of frozen sections likewise gave little information in addition to that supplied by chemical analysis of the tissue.

*Gallbladders, Bile, and Gallstones*—Whether or not gallstones developed in the cholesterol-fed guinea pigs seemed to be determined by the diet which accompanied the cholesterol. Diet A with only the basic vitamin supplements seldom produced stones. Diet B with the added supplements designated "c" and "r" usually produced stones. Guinea pigs, like human beings, often survived for long periods after stones developed.

The gallbladder of one animal which had been fed a basal diet for 212 days after cholesterol injury, and which had a normal blood count, contained a mass of large stones. Other specimens also gave evidence that stones, having once developed, are very likely to persist, even after the blood count has returned to normal.

Guinea pig bile from control animals was nearly cholesterol-free, and only faintly positive tests were ever obtained either by acetic anhydride-sulfuric acid condensation or by the digitonin precipitation technique, even from the murky contents of gallbladders that contained many stones. The stones themselves were rich in pigment and phosphate, but contained little or no sterol. Apparently, therefore, bile is not an ordinary channel for excretion of cholesterol in this species. The high pigment content of the gallstones suggests that degeneration of red cell pigment may play a part in their formation.

*Blood and Bone Marrow*—Changes in red cell count and in reticulocytes of two typical guinea pigs during and after a period of cholesterol feeding are shown in Figs 1 and 2. At the peak of the cholesterol anemia there was much evidence of new red cell formation. Reticulocyte counts were high, but there were also many cells which showed signs of beginning degeneration. The range between the concentration of NaCl required to produce beginning and that required for complete hemolysis was greater than normal. Microscopic study also indicated that the young cells with remnants of nuclear structure resisted the effects of osmotic pressure difference more successfully than mature cells.

As Table II shows, a fairly large percentage of the cholesterol in the blood of guinea pigs is held within the corpuscles. Apparently, as the cells ma-

ture in the cholesterol-fed animals, increase in lecithin fails to keep pace with increase in cholesterol. The anemia seems therefore to be associated

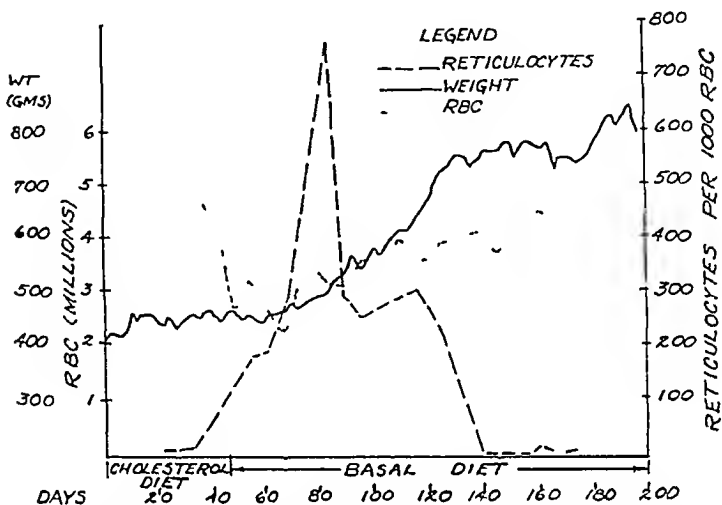


FIG 1 Weight, red blood cell count, and reticulocyte count of a guinea pig allowed to recover from cholesterol anemia

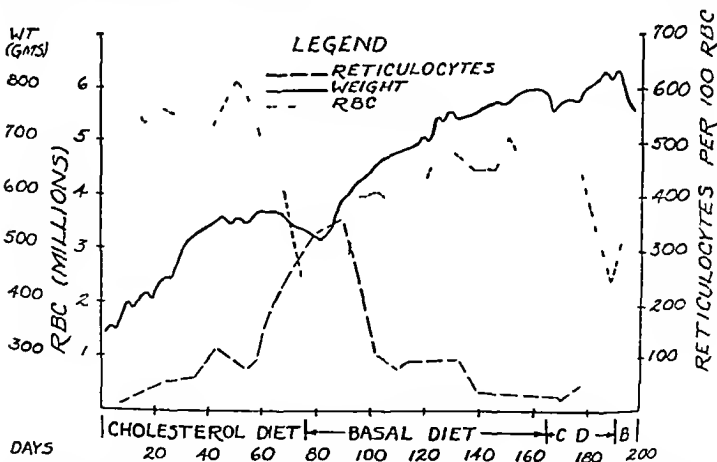


FIG 2 Weight, red blood cell count, and reticulocyte count of a guinea pig subjected to a second period of cholesterol feeding after recovery from the initial anemia

with decreased ratio of lecithin to free cholesterol and hence possibly with decreased metabolic activity

Bone marrow was observed to become extremely hyperplastic at about the time of onset of the anemia. Practically all signs of fat globules disappeared, and the entire field was crowded with immature blood cells. After the animals were removed from the cholesterol diet, evidences of hyperplasia persisted for a very long time. In the few animals killed after the red cell count had again reached 5.5 million, fat droplets were not quite normal in number.

Clearly, recovery after cholesterol injury must involve the formation, not only once, but repeatedly, of a completely new supply of red blood cells. This process must be continued at an abnormally rapid rate as long as the liver and blood cholesterol levels are abnormally high.

Many immature forms of white cells were observed in the bone marrow preparations. "Total white" counts sometimes reached 40,000 in older animals which had been removed from cholesterol diets. The possibility that some of these cells were circulating endothelial cells was not ruled out.

*Spleen*—The enlargement of the spleen of the cholesterol-fed animal apparently took place very rapidly after the red cell count reached about 4 million.

After removal of the animal from the cholesterol diet, the size of the spleen decreased slowly, in this series there were no animals in which the spleen returned to normal size. During the recovery period, moisture content decreased (79 to about 76 per cent), there occurred a slight decrease (0.67 to 0.60 per cent) in average total cholesterol content and a slight increase (1.45 to 1.81 per cent) in lecithin content. These changes may be accounted for by the decreased number of cells and the decreased quantity of cellular debris present. Certainly there was no accumulation of cholesterol ester as the functional activity of the organ became less.

Structurally, the spleens of the animals recovering from cholesterol injury were characterized by increased prominence of connective tissue framework. During acute anemia the organ was so hyperplastic and so engorged with cellular debris, endothelial cells, and red cells in all stages of development and breakdown that the underlying structure was hard to trace.

*Lungs*—The lungs of cholesterol-fed guinea pigs showed a cholesterol ester content significantly higher than in control animals. For Diet B the average figure was 0.57 per cent against a control of 0.08 per cent. Animals with evidence of nearly complete recovery from cholesterol injury had values of 0.13 and 0.17 per cent respectively. Free cholesterol remained nearer the average experimental level of 0.50 per cent than the control of 0.40 per cent. Differences were, however, hardly great enough to be significant. Microscopically, the lung sections from cholesterol-fed animals usually showed more than normal numbers of cells, presumably of endothe-

lial origin, which were entangled in the capillary network of the alveoli. These did not disappear as recovery from the anemia proceeded.

*Heart and Blood Vessels*—No cholesterol deposits have been observed in the large blood vessels, nor any sclerotic plaques.

*Adrenals*—The composition of the lipids of the adrenals in the early series of animals did not vary significantly with cholesterol feeding. Chemical analyses were therefore not made for the later series.

In some of the older animals, the inner zone of the cortex was seen to be undergoing invasion by groups of endothelial cells resembling those found in the livers. Cortical damage in these animals was suggested by the sudden and erratic weight losses, the sharp variations in red blood cell count, the sudden falling off of muscle tone, and the many otherwise unexplainable cases of overnight deaths in animals apparently near full recovery from cholesterol anemia. The percentage of cholesterol in the adrenal is normally as high as in an extremely fatty liver. Therefore, certain endothelial cells may have become sensitized to cholesterol in such a way that they took up this lipid wherever they found it. Conceivably, when cholesterol ester deposits in the liver have become exhausted, these cholesterol-seeking cells, carried by the blood stream, might bring about injury to function in a tissue which normally contains, and uses, cholesterol in high concentration. (Fig. 1 shows the weight curve and cell count for an animal of this type.)

#### DISCUSSION

The slowness with which cholesterol ester deposits in the liver are cleared out after a period of cholesterol feeding is perhaps the most striking feature of the recovery from cholesterol injury in the guinea pig. The slow rate of removal is not a characteristic of this species or this tissue alone. An unpublished and partially uncompleted study made with rats in this laboratory some years ago showed a similar picture. Dr. H. L. Gillum fed a large series of rats cholesterol until the livers, as evidenced by autopsy of a control group, were grossly fatty and enlarged. The other animals were then placed on a nearly cholesterol-free diet and sacrificed at intervals of approximately 1 week. In only eleven of the twenty animals killed about 40 days after removal from the cholesterol diet had the livers returned to approximately normal size or appearance.

The work of Rittenberg and Schoenheimer (4) is generally considered to indicate a comparatively rapid turnover of lipid in tissue. Examination of their data shows, however, that their laboratory findings are not necessarily at variance with the conclusion that cholesterol ester, once deposited in excess quantity in a tissue, is likely to remain for a long time. When these workers fed heavy water to mice, the cholesterol in the tissues had a high deuterium content, a fact which they explained on the basis of synthesis of



the cholesterol from smaller molecules. But, when they maintained an organism, such as a chick embryo, in a medium already rich in cholesterol, even when this medium was also rich in heavy water, the cholesterol in the embryo took up no deuterium. This would indicate that neither synthesis nor destruction of cholesterol occurred.

What we have been able to observe concerning the mechanism for cholesterol transport agrees reasonably well with the data of investigators who have used microscopic technique alone. Leary (5) has pictured cholesterol-laden endothelial cells in the livers of rabbits as breaking away, circulating in the blood stream, and penetrating the intima of arteries to which they transfer cholesterol ester. He believes that, normally, after considerable ester has accumulated in the wall of the artery, an irritative mechanism is set up, extra free fatty acid is attracted to the deposits, this dissolves the ester, and the deposits are removed. The last part of his hypothesis is least convincing, because the stain which he considers specific for fatty acid gives a similar color with phospholipid.

Judging from the behavior of animals with infections, there may be some resemblance in function if not in origin between these phagocytic endothelial cells and the leucocytes. As noted elsewhere (6), cholesterol-fed rats that have developed infections have always shown lowered liver cholesterol ester, with some indication of transfer of cholesterol to pus. One rat had a pus sac in the lung containing 149 mg. of total cholesterol, five-sixths of which was esterified, and had only 781 mg. of total cholesterol left in the liver. Two litter mates on the same diet had 1351 and 1768 mg. of liver cholesterol respectively. Evidence that this disappearance of liver cholesterol may take place rapidly has also been presented (6). A rapid fall in blood cholesterol associated with the development of infection has been noted in human beings (7). A few guinea pigs which developed abscesses of the mammary gland had markedly lowered liver and blood cholesterol. Sperry and Stoyanoff (8) found that in rats infected with paratyphoid the cholesterol ester in the liver decreased during the time when the free cholesterol of the carcass was increasing. Inflammatory processes with leucocyte mobilization are wide-spread in this type of infection.

Bovd concluded from two studies (9) that effective resistance to infection is associated with increase in the free cholesterol and lecithin of the leucocytes. He explained this as due to high metabolic activity of these cells, on the basis of evidence presented by Bloor and his coworkers (10).

That phagocytosis is one main mechanism for removing cholesterol ester deposits from tissues seems, therefore, reasonably clear. Apparently, also, specially adapted or sensitized cells may be developed for this function in emergency. In the animal not suffering from bacterial infection these cells may possibly be developed from the endothelial cells in the liver or, as some of our slides suggest, possibly from bone marrow and spleen as well. In

active infections, polymorphonuclear leucocytes appear to take up extra cholesterol, or to multiply so greatly that their part in the process becomes evident

The ultimate fate of the cholesterol is less clear. Neutralization of the effect of certain bacterial toxins must involve the use of cholesterol, or perhaps the presence of extra cholesterol in the leucocytes aids in lysis of engulfed bacteria. Conceivably, cholesterol may be freed in quantity and may then destroy not only the bacterial cells but also the leucocytes, since in the process of pus formation the leucocytes die.

Sperry observed (11) that serum contains an enzyme capable of synthesizing cholesterol ester from free cholesterol and fatty acid. This indicates a normal mechanism for limiting the percentage of free cholesterol in circulating blood. Perhaps the critical level may be associated with the lecithin to cholesterol ratio and with phenomena of cell membrane equilibria. Sperry's demonstration that the action of the enzyme is reversed in the presence of bile salts would at least suggest an explanation of the high plasma values for free cholesterol associated with liver injury. Action of the bile salt on surface tension may even facilitate penetration of cholesterol into blood corpuscles. (The presence of bile pigment in the blood of anemic guinea pigs has been demonstrated in this laboratory.)

The low cholesterol content of guinea pig bile suggests a species difference in the mechanism for its disposal. In human beings bile is so nearly a saturated solution of cholesterol that formation of cholesterol crystals around nuclei of precipitated bile pigment is the usual story in gallstone formation. Other species variations in the channels of excretion and in ease of disposal of excess cholesterol undoubtedly exist. Carnivorous animals fed cholesterol apparently mobilize it readily and do not store it in the liver in excessive amounts (12). The rat stores the ester, but it is comparatively non-toxic. In rabbits cholesterol is transferred from fatty livers to irritating but perhaps relatively inert deposits in the arterial walls. What happens in human beings is not entirely clear, but lesions involving cholesterol deposition are relatively common in human pathology. The guinea pig presents a dramatic picture of cellular destruction and anemia. On the other hand, malignant growths, which are characterized by a high cholesterol content, seldom are seen in guinea pigs. These are common in species exhibiting moderate difficulty in handling cholesterol, for example, the rat and man. Clarification of the function of cholesterol in cellular metabolism remains a challenge to the investigator.

#### SUMMARY

From a study of guinea pigs recovering from the injury produced by a period of cholesterol feeding, the following conclusions apparently may be drawn

the cholesterol from smaller molecules. But, when they maintained an organism, such as a chick embryo, in a medium already rich in cholesterol, even when this medium was also rich in heavy water, the cholesterol in the embryo took up no deuterium. This would indicate that neither synthesis nor destruction of cholesterol occurred.

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# CONCERNING THE CHARACTERIZATION OF POSSIBLE CORTICAL HORMONE METABOLITES IN URINE\*

By LOUIS F FIESER, MELVIN FIELDS, AND SEYMOUR LIEBERMAN

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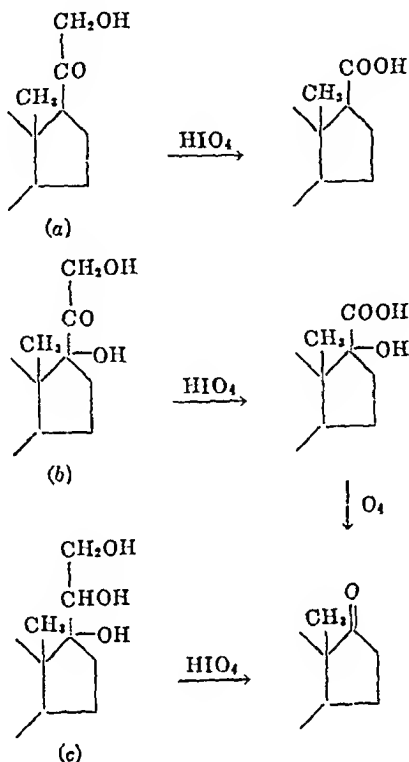
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Investigations of the nature of urinary steroids by both chemical (2, 3) and biological (4-7) methods have afforded certain tentative indications of the presence of substances having distinguishing characteristics associated with hormones of the adrenal cortex or of products of the metabolism of these hormones. Methods for either the determination or the selective fractionation of any such substances present in urinary extracts should advance the knowledge of intermediary hormone metabolism and might be of considerable clinical significance. The distinguishing oxygen function at  $C_{11}$  is present in some but not all of the recognized cortical steroids, and no known property associated with this function lends itself to either separation or analysis. A more general structural characteristic is the presence of the 3-carbon unit  $C_{17}$ - $C_{20}$ - $C_{21}$  in a uniquely high state of oxidation and comprising what may be described as a triose or desoxytriose unit. This paper describes orienting attempts to characterize possible cortical hormone metabolites in urine based upon the sugar-like nature of the side chain.

## *Oxidation with Periodic Acid*

Among the known  $C_{21}$  cortical steroids, all those possessing characteristic biological activity, as well as certain of the inactive substances, have either the ketol grouping (a) or the 17-hydroxy ketol (dihydroxyacetone) grouping (b), while the glycerol grouping (c) is present in some of the inactive companion substances. All but two of the recognized  $C_{21}$  cortical steroids conform to one of the three patterns indicated. These structures are all susceptible to glycol cleavage with either periodic acid or lead tetraacetate. The ketols (a) are converted by these specific reagents into etio acids, the substances with the dihydroxyacetone grouping (b) are oxidized initially to  $\alpha$ -hydroxy acids and then, with excess reagent, may yield 17-ketosteroids, and compounds possessing the glycerol side chain (c) afford 17-ketosteroids directly.

\* For reviews and references see Reichstein and Reichstein and Shoppee (1)



*Titration with Periodic Acid*—The specificity of the glycol cleavage reaction, at least as applied to pure compounds, suggested the possibility that titration of urinary extracts with a suitable reagent might afford an index of the amount of cortical steroid metabolite present. The micro-method of Rappaport *et al* (8) for the determination of certain hexoses with periodic acid was adapted to the problem of estimating water-insoluble steroids in pigmented urinary extracts by employing methanol as the solvent and determining the unutilized reagent by potentiometric titration with standard arsenious oxide solution. Trials with six 1 day non-ketonic steroid fractions of human urines were discouraging, for periodic acid was consumed in amounts far in excess of that which would be required if the entire fraction were composed of glycol components. Indeed it appeared that substances must be present that are capable of reducing periodic acid beyond the stage of iodic acid, the normal stopping point in the oxidation of glycols and glycerols. Williams and Woods (9) have shown that iodic acid is a powerful oxidizing agent for many different types of organic substances, even in the cold.

*Oxidation of Non-Ketonic Steroid Fractions*—Cortical steroid metabolites possessing the glycerol grouping (c) or the dihydroxyacetone grouping (b) should be convertible, by direct glycol cleavage or by cleavage and subsequent oxidation of the  $\alpha$ -hydroxyetio acids, into 17-ketosteroids. The production of 17-ketosteroids from non-ketonic steroid fractions by oxidation with periodic acid or lead tetraacetate would thus constitute evidence of the presence of substances probably derived from the adrenal cortex. Since the tertiary hydroxyl group at C<sub>17</sub> is subject to ready elimination under the dehydrating action of mineral acids, acid-hydrolyzed urine undoubtedly is an unfavorable starting material for an investigation along the lines indicated. In the absence of material more favorable for study in quantity, experiments designed to determine whether glycol cleavage can give rise to the production of 17-ketosteroids were carried out with the non-ketonic neutral fraction derived from commercially processed acid-hydrolyzed pregnancy urine.

TABLE I  
*Oxidation of Non-Ketonic Fraction of Pregnancy Urine*

Experiment No	Sample No	Oxidant	Ketonic fraction produced	
			Weight	Yield
	gm		gm	per cent
1	I 50	HIO <sub>4</sub>	1.07	2.1
2	Ia 30	"	1.80	6.0
3	II 30	"	0.70	2.3
4	" 90	"	4.14	4.6
5	" 30	Pb(OCOCH <sub>3</sub> ) <sub>4</sub>	0.61	2.0

The results of five oxidation experiments are listed in Table I. Sample I, used in Experiment 1, had been reextracted with Girard's reagent to remove traces of ketones, a 50 gm sample was oxidized with 5.0 gm of crystalline periodic acid (dihydrate) in methanol (36 hours at 25°). This afforded 1.2 gm of acidic material and a ketonic fraction amounting to 3.0 gm, reextraction of the latter fraction with Girard's reagent gave 1.07 gm of dark brown, glassy material having a value in the Callow-Zimmermann colorimetric determination for 17-ketosteroids of 140 mg of androsterone equivalents. No precipitation was observed on treatment of portions with semicarbazide acetate and with digitonin, and no crystalline eluates were obtained when the material as such was chromatographed on alumina. Another portion (208 mg) was separated with phthalic anhydride and the alcoholic fraction (59 mg) and non-alcoholic fraction (107 mg) were chromatographed on magnesium sulfate-Celite, but without success.

In Experiment 2, 100 gm of Sample I were separated with phthalic

anhydride and the alcoholic fraction (Sample Ia, 30 gm) was oxidized with periodic acid. The material extracted with Girard's reagent (1.8 gm) was processed with phthalic anhydride and afforded 830 mg of alcoholic ketonic steroids having a Callow value of 93 mg (no precipitate with digitonin).

Sample II was obtained by processing the crude extract (615 gm) from about 800 liters of human pregnancy urine as follows. The alcoholic fraction was segregated through the half phthalate and the material recovered (190 gm) was treated three times with Girard's reagent to remove all traces of ketones from the residual neutral non-ketonic alcoholic fraction (154 gm). Three large scale glycol oxidation reactions were conducted and ketonic fractions were obtained in 2 to 4 per cent yield (Table I), but no crystalline products could be isolated. In Experiment 4 the reaction mixture afforded 1.46 gm of acidic material and 4.14 gm of an oily ketonic fraction, and the latter had a colorimetric value of 870 mg of androsterone equivalents. Seven chromatograms of the ketonic fraction, involving a total of 421 eluates, failed to afford crystalline products.

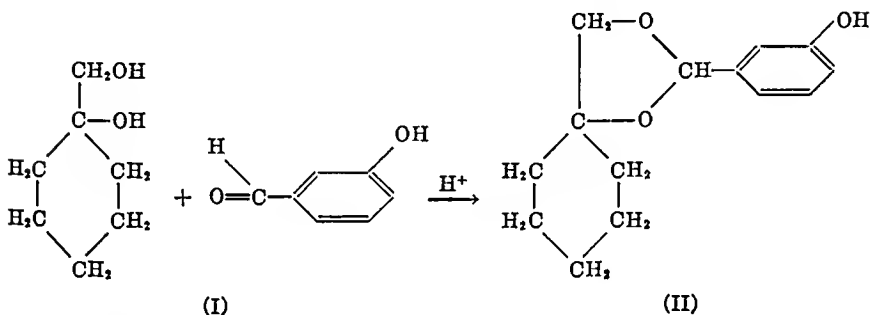
*Ketosteroid Assay Following Periodic Acid Oxidation*—Several ketonic fractions from acid-hydrolyzed human urines were oxidized with periodic acid in methanol, but no increase in 17-ketosteroid content was noted and in several instances there was a distinct drop in the Callow value. On the other hand, the oxidation of synthetic  $\Delta^4$ -pregnenetriol-17 $\alpha$ ,20,21-one-3 (10 mg) with 2 parts of periodic acid dihydrate resulted in an increase in Callow value from 4.1 to 14.1 mg (theoretical increase, 8.2 mg). Such other early experiments in the direction of the development of an assay method based upon the 17-ketosteroid content after glycol cleavage were of a preliminary nature and are superseded by the more extensive study reported in a paper by Talbot and Etingon (10), with whom we have exchanged information.

### *Acetal Formation*

A second possible method investigated for the characterization of cortical steroid metabolites having a highly hydroxylated side chain was by condensation with an aldehyde so substituted that the expected acetal derivatives of any 1,2- or 1,3-glycols present could be selectively extracted from a mixture with acid or with base. The steroids of types (b) to (c), listed above, should all afford cyclic acetals, and by analogy to known compounds these should be stable to alkali and possibly subject to cleavage to the original steroids under mild conditions of acid hydrolysis.

In exploratory experiments several acetals were prepared by the condensation of simple model glycols with variously substituted benzaldehydes. The technique introduced by Salmi (11) for the preparation of otherwise difficultly accessible ketals proved advantageous, and the best results were

obtained by using benzene or ethyl acetate as solvent and slowly distilling the mixture of components, solvent, and catalyst to remove the water formed in the equilibrium reaction. A trace of *p*-toluenesulfonic acid effectively catalyzed the reactions, but all attempts to find non-acidic catalysts which would be more sparing of the sensitive C<sub>11</sub>- and C<sub>17</sub>-hydroxyl groups of the cortical steroids were unsuccessful. Thus calcium chloride, ferric chloride, ammonium chloride, and ammonium sulfate (12) all proved unsatisfactory. The model glycols investigated were ethylene glycol, 1,2-propylene glycol, and 1-hydroxymethylcyclohexanol-1 (I), and the aldehydic components were *m*- and *p*-nitrobenzaldehyde, terephthalaldehyde methyl ester, *o*-, *m*-, and *p*-hydroxybenzaldehyde. Thus a typical example is the preparation of the acetal II







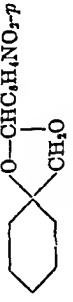
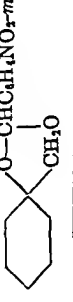
**Typical Procedure**—In a representative preparation a solution of 2.0 gm of *p*-nitrobenzaldehyde, 2.0 cc of redistilled ethylene glycol, and a few crystals of *p*-toluenesulfonic acid in 300 cc of dry benzene was refluxed under a special take-off condenser (13) of the type used by Salmi (11) at such a rate that at the end of 2 hours about three-fourths of the solvent had been distilled. 200 cc of ethyl acetate were added and the solution was washed with water, dried over sodium sulfate, and evaporated *in vacuo*. The residue on cooling was a white crystalline solid, m p 91–94°, consisting of the very nearly pure acetal (it depressed the melting point of *p*-nitrobenzaldehyde). The analytical sample was recrystallized from acetone and melted at 89–89.5°. The acetal was hydrolyzed readily by hot dilute acetic acid and afforded *p*-nitrobenzaldehyde. Equally satisfactory results were obtained with the use of ethyl acetate as solvent and with potassium acid sulfate as the catalyst, but with the neutral agents cited above no acetal formation was noted.

**Properties and Analyses of Model Acetals**—The acetals listed in Table II were prepared by the procedure outlined, with the yields noted.

The nitrobenzaldehydes afford glycol acetals in good yield, but there seems to be little promise of developing a procedure of separation based



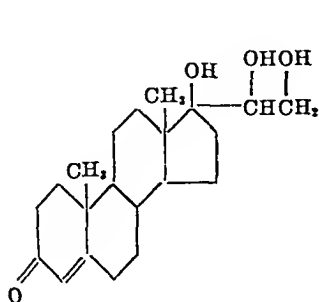
TABLE II  
*Model Acetals*

Formula	Components	Yield per cent	C		H	
			Calcu- lated per cent	Found per cent	Calcu- lated per cent	Found per cent
	HOCH <sub>2</sub> CH <sub>2</sub> OH, <i>p</i> -NO <sub>2</sub> C <sub>6</sub> H <sub>4</sub> CHO	98.5	55.38	55.41	4.65	4.73
	HOCH <sub>2</sub> CH <sub>2</sub> (CH <sub>2</sub> )OH, <i>p</i> -NO <sub>2</sub> C <sub>6</sub> H <sub>4</sub> CHO	80	57.38	57.73	5.30	5.19
	HOCH <sub>2</sub> CH <sub>2</sub> OH, <i>m</i> -NO <sub>2</sub> C <sub>6</sub> H <sub>4</sub> CHO	70	55.40	55.62	4.61	4.65
	HOCH <sub>2</sub> CH <sub>2</sub> (CH <sub>2</sub> )OH, <i>m</i> -NO <sub>2</sub> C <sub>6</sub> H <sub>4</sub> CHO	75	57.38	57.44	5.30	4.92
	I, <i>p</i> -NO <sub>2</sub> C <sub>6</sub> H <sub>4</sub> CHO	65	63.81	63.99	6.51	6.29
	I, <i>m</i> -NO <sub>2</sub> C <sub>6</sub> H <sub>4</sub> CHO	55	63.81	64.04	6.51	6.32

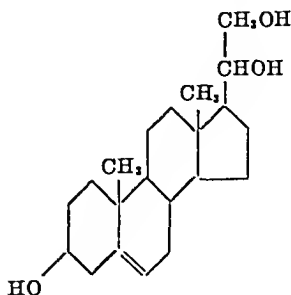
	$\text{HOCH}_2\text{CH}_2\text{OH}$ , $p\text{-CH}_2\text{OOC C}_6\text{H}_4\text{CHO}$	B p 168-173, 13 mm	67	63 45	63 60	57 77	5 84
	$\text{HOCH}_2\text{CH}(\text{CH}_3)\text{OH}$ , $p\text{-CH}_2\text{OOC C}_6\text{H}_4\text{CHO}$	" 125-130, 15 "	57	64 80	64 78	6 34	6 43
	$\text{HOCH}_2\text{CH}(\text{CH}_3)\text{OH}$ , $o\text{-HO C}_6\text{H}_4\text{CHO}$	" 155-156, 25 "	24	66 62	66 30	6 71	6 79
	$\text{HOCH}_2\text{CH}(\text{CH}_3)\text{OH}$ , $m\text{-HO C}_6\text{H}_4\text{CHO}$	" 142-146, 2 "	59	66 62	66 23	6 71	6 98
	I, $o\text{-HO C}_6\text{H}_4\text{CHO}$	" 145-149, 2 "	46	71 73	71 96	7 74	7 82
	I, $m\text{-HO C}_6\text{H}_4\text{CHO}$	" 185, 25 mm, m p 73-74	76	71 73	71 91	7 74	7 96
	I, $p\text{-HO C}_6\text{H}_4\text{CHO}$	B p 180-186, 2 mm, m p 76-80	50	71 73	71 39	7 74	7 96

upon reduction and extraction of the amine derivative with acid, for this probably would result in cleavage of the acetal linkage. The free phthalaldehydic acids appeared unpromising as reagents because of their sparing solubility in non-aqueous solvents and because of the opportunity for ester formation by interaction with steroid hydroxyl groups. Terephthalaldehyde methyl ester could be condensed satisfactorily with model glycols, but such a reagent would have the disadvantage of affording opportunity for the damaging of alkali-sensitive steroids in the saponification of the esterified product prior to extraction. The phenolic reagents are free from the limitations enumerated, and *m*-hydroxybenzaldehyde gave better yields than the isomers and thus proved to be the most satisfactory aldehydic reagent for the purpose at hand.

**Acetal Separation of a Model Glycol**—As a means of evaluating the scheme of separation an attempt was made to isolate 1-hydroxymethylcyclohexanol-1 from a mixture of the glycol (10.3 gm) with mineral oil (50 cc). A solution of the mixture in 800 cc of benzene containing 30 mg of *p*-toluenesulfonic acid and 10 gm of *m*-hydroxybenzaldehyde was refluxed for 2 hours, during which time 400 cc of distillate were allowed to collect. The residual solution was extracted with 2% sodium hydroxide and the alkaline extract was cooled to 0°, neutralized carefully with dilute acetic acid solution, and extracted with ether. Distillation of the material recovered from the washed and dried ethereal extract afforded 12.4 gm (66 per cent) of the acetal as a viscous oil (b.p. 174° at 1 mm) that crystallized on standing (m.p. 73–74°). For hydrolysis, a solution of 7.3 gm of the acetal in 30 cc of 50 per cent acetic acid was heated for 3 hours on the steam bath. The solvent was removed *in vacuo* and the residue dissolved in ether and extracted with 2% sodium hydroxide. The neutral fraction on recrystallization from ether gave 1.3 gm of 1-hydroxymethylcyclohexanol-1, representing a 22 per cent over-all recovery.



(III)



(IV)

**Acetal Separation of Model Steroids**—One of two compounds of the steroid series investigated as models of cortical hormone metabolites was

$\Delta^4$ -pregnenetriol-17 $\alpha$ ,20,21-one-3 (III) This was prepared by known reactions, starting with the Oppenauer oxidation of  $\Delta^5$ -17-ethynylandrostenediol<sup>1</sup> to  $\Delta^4$ -17-ethynylandrostenol-17-one-3 (14), yield 66 per cent) Reduction of the acetylenic substance in pyridine with Raney's nickel or with 2 per cent palladium on calcium carbonate (15) afforded  $\Delta^4$ -17-vinylandrostenol-17-one-3 in 70 per cent yield, the hydrogenation proceeded smoothly at room temperature and pressure and came to an abrupt stop at the desired stage The triol III was obtained in 40 per cent yield by hydroxylation with osmium tetroxide (16) and reductive hydrolysis of the osmic ester with sodium sulfite, the purified substance melted at 234.5–235.5°, corrected The volatile osmium tetroxide was conveniently stored in the form of a standard solution in carbon tetrachloride and kept in a flask with a tightly fitting ground glass stopper

On treatment of the triol III in benzene solution with *m*-hydroxybenzaldehyde and a trace of *p*-toluenesulfonic acid, 75 per cent of the substance was converted into alkali-soluble material, presumably the acetal The neutral fraction was not the unchanged triol but an uncrystallizable oil, probably resulting from the dehydration of III The alkaline extract was cooled to 0° and carefully acidified with dilute acetic acid and extracted with ether The crude acetal recovered from the ethereal extract was submitted to very mild hydrolysis with dilute acetic acid but the resulting neutral fraction was an oil which failed to crystallize Experiments with the pure triol III then indicated that the difficulty probably is in the very great sensitivity of the substance to the dehydrating action of acids Refluxing the triol in benzene solution with a trace of *p*-toluenesulfonic acid for 2 hours resulted in the conversion of 40 per cent of the material into a resinous product Treatment with dilute acetic acid or with anhydrous copper sulfate also resulted in considerable alteration Reichstein and coworkers (17, 18), working with compounds similar to III but of the C<sub>17</sub>- $\beta$  configuration, were able to effect satisfactory conversion to the acetone derivatives with the use of anhydrous copper sulfate as catalyst, but this case is different from the present one because a large excess of the carbonyl compound (acetone) could be employed

The second steroid studied was  $\Delta^5$ -pregnenetriol-3 $\beta$ ,20,21 (IV), prepared by aluminum isopropoxide reduction of  $\Delta^5$ -3-hydroxy-21-acetoxypregnenone-20<sup>1</sup> and hydrolysis, according to Steiger and Reichstein (18) This substance has no tertiary hydroxyl group at C<sub>17</sub> and should be less sensitive to dehydration than III A solution of 100 mg of IV (stereoisomeric mixture, m p 201–210°), 100 mg of *m*-hydroxybenzaldehyde, and 20 mg of *p*-toluenesulfonic acid in 12 liters of dry benzene was refluxed

<sup>1</sup> For supplies of this material we are greatly indebted to Dr Erwin Schwenk, the Schering Corporation, and to Dr C R Scholz, the Ciba Pharmaceutical Products, Inc

for 18 hours, during which time about half of the solvent was allowed to distil. Partition with alkali afforded an only neutral fraction amounting to 60 mg, the alkali-soluble acetal fraction was acidified with excess hydrochloric acid to effect hydrolysis, and the resulting neutral material yielded, after recrystallization, 20 mg of the original  $\Delta^5$ -pregnenetriol-3( $\beta$ ), 20, 21 (IV), m p 205-215°

These orienting experiments are not very encouraging. The triol III, with a tertiary  $\alpha$ -hydroxyl at C<sub>17</sub>, is subject to alteration (probably dehydration) under the conditions required for the formation and hydrolysis of the acetal derivative. In the case of the triol IV, which has no tertiary hydroxyl groups, conversion to the alkali-extractable acetal of *m*-hydroxybenzaldehyde and regeneration of the original steroid can be realized, but the yields in both steps are far from quantitative. The acetal separation procedure was tried on a sample of extract of the acid-hydrolyzed pregnancy urine described above, but a twice processed "glycol fraction" afforded no crystalline products.

We are greatly indebted to Dr C P Rhoads and Dr K Dobriner, of Memorial Hospital, New York, for supplies of hormone fractions, for colorimetric determinations, and for their general cooperation. This project was conducted in part under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Memorial Hospital.

#### SUMMARY

Two possible methods are suggested for characterizing cortical hormone metabolites that may appear in the urine. Both methods are based on the sugar-like nature of the highly oxygenated side chain. One, involving glycol cleavage with periodic acid or lead tetraacetate, offers some promise. The other, involving conversion of a 1,2- or 1,3-glycol to an alkali-extractable acetal derivative, is subject to the serious limitation that the conditions of acid catalysis required for the formation and cleavage of the acetals are sufficiently severe to cause some elimination of the acid-sensitive C<sub>11</sub>- and C<sub>17</sub>-hydroxyl groups.

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# DIGESTION OF RAW STARCH\*

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## PLATE 1

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The resistance of uncooked starches to enzymic digestion *in vitro* has been variously attributed to the protection of the granule by a surface layer of amylopectin (Weichsel (1)), protein (Gortner and Hamalainen (2)), fat (Taylor and Sherman (3)), hemicellulose (Schroyer and Thomas (4)), or relatively impermeable metallic complexes with phosphoric acid (Lynst-Zwicker (5)). Experiments to remove the protecting layer by specific enzyme digestion have been either unsuccessful or inconclusive. Yet in 1879 it was recognized by Brown and Heron (6) that raw starch could be digested. Attempts have also been made to correlate the size of the starch granules with their susceptibility to breakdown by enzymes, but Stamberg and Bailey (7) have concluded that there is no correlation between such susceptibility and either granule size or phosphorus content. Blish, Sandstedt, and Mecham (8) have proposed that a special raw starch-digesting factor is involved, probably an enzyme associated but not identical with  $\alpha$ -amylase.

In the recorded data, the rate of digestion of raw starch has always been shown as very slow. Thus Stamberg and Bailey (7), whose attempts at rapid digestion were relatively successful, found about 10 per cent conversion of starch to sugar after 24 hours exposure to an  $\alpha$ -amylase preparation. The speed and completeness of the enzymic breakdown of raw starch as observed *in vitro* have been very much less than in the living animal.

It is well recognized that rapid and relatively complete conversion of cooked starch to sugars requires two distinct enzymes,  $\alpha$ -amylase and  $\beta$ -amylase. In the course of work in this laboratory the relative efficiency of  $\alpha$ -amylase from several sources was tested, with the intention of supplementing therewith the  $\beta$ -amylase of unmalted wheat for the digestion of starch in wheat flour. In these experiments it became apparent that although pancreas tissue and cultures of *Aspergillus oryzae* are both admittedly rich in  $\alpha$ -amylase (and were found so to be) they were not inter-

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changeable as supplements to wheat  $\beta$ -amylase. They did, however, supplement each other.

It occurred to us that in employing a crude enzyme preparation of animal origin we might be including some factor corresponding to that which makes the utilization of raw starch by animals an efficient process. In any event, we were including most of the factors proposed by other workers as possibly responsible for the digestion of raw starch. The mixture of *Aspergillus* and pancreas amylases was therefore tried on wheat flour with the result that the raw starch was rapidly converted to a mixture of glucose

TABLE I

*Acceleration of Raw Starch Digestion by Flour Extracts and Salts*

The system consisted of 0.8 gm. of mold bran, 0.08 gm. of commercial pancreatin, and starch and additions shown below, in a volume of 100 cc., at pH 4.8 (0.05 M acetate buffer). It was incubated and stirred slowly for 40 hours at 40°.

Addition	Total reducing capacity*	
	Wheat starch, 3.2 gm.	Extracted flour †† 3.2 gm.
	m eq.	m eq.
No addition	1400	1650†
Water extract from 3.2 gm. flour	1890	2630
“ “ “ 10 “ “	3130	3360
“ “ “ 10 “ “ heated 1 hr	3200	
“ “ “ 10 “ “ ashed	3140	3310
Gluten from 10 gm. flour	1360	
Calcium chloride, 0.05 gm.	3170	3330
Sodium “ 0.02 “	2950	
Calcium nitrate, 0.04 “	3010	

\* Reducing capacity is expressed as miliequivalents of ferricyanide per 100 gm. of starch acted upon, as explained in the description of analytical methods.

† Flour after the extraction with water and the water extract were prepared by suspending 10 gm. of patent flour in 75 cc. of water and centrifuging after about 10 minutes.

†† The unextracted flour gave corresponding reducing capacity of 3350.

and maltose. However, more experiments indicated that purified starch, from either corn or wheat, was not hydrolyzed as rapidly as the starch present in flour. On the addition of a water extract of wheat flour to the system containing pure starch, the deficiency was made up and the pure starch was digested as fast as starch in the flour. Further experiments showed that the ash of flour was as effective as the extract. Analyses of the ash showed that the constituents active in this connection were chloride and calcium ions, as shown in Table I. The activation of both ions on amylolysis is well known.

The enzyme mixture ultimately adopted to exhibit the disintegration of raw starch consisted of commercial pancreatin or freshly ground hog pancreas and a culture of *Aspergillus oryzae* grown on wheat bran. The latter is commonly referred to as "mold bran" and has found considerable application in the fermentation industries as a substitute for malt.<sup>1</sup> The experiments reported here were all carried out in the presence of toluene, and with continuous stirring. The methods used to determine the extent of hydrolysis are given in the next section.

The hydrolysis of starch present in uncooked wheat flour produced by a mixture of mold bran and pancreas enzymes is shown in Table II. The sugar formed is a mixture of glucose and maltose, in which the former sugar greatly predominates.

TABLE II

*Per cent of Uncooked Starch in Patent Flour Converted to Glucose and Maltose*

The system consisted of 3.2 gm of wheat flour, 0.8 gm of mold bran, and 0.1 gm of a sample of commercial pancreatin in 100 cc of 0.05 M acetate buffer, pH 4.7, at 40° under toluene with slow stirring.

Time	Ratio glucose to maltose	Starch converted
<i>hrs</i>		<i>per cent</i>
23.5	3.8	58.5
47.0	3.5	96.3
96.0	3.7	86.3

The flour contained 13.1 per cent moisture, 12.2 per cent protein, 1.1 per cent fat, and 0.5 per cent crude fiber, so the amount of dry starch per 100 cc was equal to or less than 2.34 gm. The percentage of starch conversion is calculated on this amount because of the difficulty of determining the true starch content of flour accurately.

The effect of the mixture of *Aspergillus* and pancreas enzymes (in the presence of calcium chloride) on the amount and kind of sugars formed from wheat starch is compared in Table III with the much smaller effect of each ingredient alone. Another series of experiments in which the rates of hydrolysis are comparable to each other (but not to those of Table III because of the different temperature) is shown in Table IV. In this series it appears that the pancreas enzymes supplement the action of both malt and *Aspergillus* amylases on cooked starch. On the raw starch, however, the supplementing effect of the pancreas preparation is much more marked with *Aspergillus* than with malt amylases.

<sup>1</sup> The authors express their sincere thanks to Professor Ellis I. Fulmer of the University of Iowa, to Dr. Leo M. Christensen of the University of Nebraska, and to Mr. George A. Jeffreys of Salem, Virginia, for generous quantities of mold bran, to Dr. Victor Conquest of Armour and Company, Chicago, for preparations of pancreatin, and to Mr. J. Leslie Hale of Swift and Company, San Francisco, for fresh pancreas.

In all of the foregoing examples the sum of the maltose and glucose formed from starch by the mold bran and pancreas mixture appears to be

TABLE III

*Per Cent of Uncooked Wheat Starch Converted to Glucose and Maltose*

The system consisted of 3.2 gm of wheat starch,\* and the quantities of enzyme mixture and calcium chloride shown below, in 100 cc of 0.05 M acetate buffer, pH 5.2, at 40° under toluene with slow continual stirring

Enzyme mixture added gm per 100 cc	CaCl <sub>2</sub> added gm per 100 cc	Ratio glucose to maltose formed in			Starch converted in		
		4 hrs	19 hrs	43 hrs	4 hrs	19 hrs	43 hrs
					per cent	per cent	per cent
Water extract of 1 gm mold bran (centrifuged)	None	0.5	1.4	3.0	35	32	37
	0.03	0.5	0.9	5.8	35	44	50
Commercial pancreatin, 0.1 gm	None	0.1	1.2	2.3	11	11	12
	0.03	0.2	0.6	0.8	47		73
Mold bran (as extract) 1 gm + pancreatin 0.1 gm	None	1.1	2.3		71	90	
	0.03	0.9	1.8		83	101	

\* The starch contained 9.98 per cent water and 1.10 per cent protein (N × 6.25), so the amount of dry starch used was 2.84 gm per 100 cc. The percentage of starch conversion is calculated on this amount.

TABLE IV

*Comparison of Behavior of Raw and Cooked\* Wheat Starch*

The system consisted of 3.2 gm of wheat starch, the enzyme mixture shown below, and 0.05 gm of calcium chloride in 100 cc of 0.05 M acetate buffer at pH 5.2. Analyses were made after 3 hours at 50°.

Enzyme mixture	Total reducing capacity		Starch converted	
	Boiled starch	Raw starch	Boiled starch	Raw starch
	mg	mg	per cent	per cent
1.0 gm ground malt†	1843	978		
1.0 " " " † + 1.0 gm ground pancreas	2605	1385	92	44
0.5 gm whole mold bran	2348	1600		
0.5 " " " " + 1.0 gm ground pancreas	2743	3020	94	101

\* Cooked starch was prepared by boiling a 4 per cent suspension of the raw starch in water for 5 minutes.

† The values reported for malt have been corrected for the starch present in the malt by a blank experiment without added starch.

quite close to that theoretically possible, and thus higher by 10 or 15 per cent than the yield of sugars obtained ordinarily with cooked starch.

By using large proportions of enzyme to substrate, however, Somogyi (9) and Stark (10) have hydrolyzed cooked starch completely to sugars, and have also obtained glucose thereby

The breakdown of granules of corn, wheat, and potato starch by a pancreatin-mold bran mixture at pH 5.2 in the presence of calcium chloride is shown in a series of photographs taken at intervals over periods up to 60 hours (Fig. 1). The digestions were made at 45°, but were slower than normal because there was no stirring

### *Analytical Methods*

The increase in the total reducing capacity of the solution was determined with alkaline ferricyanide. At the same time the reducing power of the solution toward a weakly acid solution of copper acetate was determined. The solution was then fermented with yeast and the same determinations made again.

After incubation the entire starch-enzyme mixture (usually 75 or 100 cc) was treated with 10 cc of 0.1 N sodium hydroxide solution and filtered. The residue of debris and undigested starch was washed with water, and the filtrate and washings were diluted to a suitable known volume, generally 250 or 500 cc, depending on the amount of sugar expected. A 5 cc portion of the diluted solution was then pipetted into 10 cc of 0.05 N alkaline ferricyanide solution, prepared as described in "Cereal laboratory methods" (11). The subsequent procedure for determining the ferricyanide reduced was that given in detail in this reference. The increase in total reducing capacity (TRC) has been expressed as milliequivalents of ferricyanide reduced per 100 gm of starch or flour used in the test. Under these conditions complete conversion of 100 gm of starch to glucose would give a value for total reducing capacity of 3450, whereas complete conversion to maltose would give a value of 2640.

Glucose (G) was measured as the difference between "apparent glucose," as determined by the acid copper procedure of Tauber and Kleiner (12), before and after fermentation of a 25 cc sample of the (diluted) filtrate with bakers' yeast for 3 hours at 35°, as directed in detail by Stark and Somogyi (13).

Maltose (M) was calculated as  $C_{12}H_{22}O_{11} \cdot H_2O$  on the assumption that the fermentable reducing capacity (FRC) (i.e., the difference between the total reducing capacity (for ferricyanide) before and after fermentation) is the sum of the reducing capacity due to maltose and that due to glucose (GRC). This assumption is justified in the case of the mixture of enzymes by the fact that all of the starch taken is ultimately accounted for as maltose and glucose. If fermentable dextrins were present, the observed total reducing capacity would naturally be less, and the total sugar found

could never account for all of the starch. It is not meant to imply that when the starch is incompletely hydrolyzed no fermentable dextrin can be present. The amount of glucose equivalent to 1 millicquivalent of ferricyanide is determined by reducing the ferricyanide with known quantities of pure glucose. The amount of maltose required to reduce 1 millicquivalent of ferricyanide is expressed by the coefficient  $C_M$ , which is determined by measuring the reduction of the alkaline ferricyanide solution with known amounts of pure maltose. Thus the maltose content equals  $(FRC - GRC)C_M$ . The total starch converted to maltose and glucose per 100 gm of material is then  $0.9 (M + G)$  or  $0.9 ((FRC - GRC)C_M + G)$ .

*Further Observations*—If either material is heated for 30 minutes at  $100^\circ$ , rapid digestion no longer takes place, indicating that heat-labile factors, presumably enzymes, necessary to the digestion of raw starch are present in both mold bran and pancreas. On the other hand, the rate of digestion with the quantities of enzyme described here is appreciably greater at  $50^\circ$  than at  $40^\circ$ . Over a period of 10 to 12 hours at  $55^\circ$ , the rate is roughly the same as at  $50^\circ$ , but at  $60^\circ$  it is again appreciably less than at  $50^\circ$ .

The conversion of raw starch to glucose and maltose is not especially sensitive to pH in the presence of calcium chloride. Tests at pH 4.6 and 6.0 gave practically the same quantity of reducing sugars (TRC) in 6 hours at  $40^\circ$ , whereas at pH 5.2 and 5.8 the quantity was about a third more. The optimum pH is in the neighborhood of 5.2. In the absence of calcium chloride this optimum is more critical.

The proportion of starch to liquid was found to influence the rate of hydrolysis considerably. Thus with 4 gm of starch in 100 cc, 83 per cent of the starch was converted to sugars in 4.5 hours at  $50^\circ$ , with 8 gm of starch, the same result was observed, but with 9.2 gm of starch per 100 cc, only 70 per cent of the starch was converted to sugars in the same time, although in this case 6 times the quantity of mold bran had been used. Further variations are shown in Table V. The digestion of pure starch is less adversely affected than is the digestion of the starch in wheat flour when the concentration of starch is increased in the system. An amylolytic inhibitor, such as Kneen and Sandstedt (14) have found in flour, could explain the difference in behavior.

It is probable from the composition of the sugar mixture formed that a maltase plays an important part in the breakdown of raw starch. The mold bran appears to be the principal source of this maltase, as it alone of the ingredients of the mixture showed a marked maltose-splitting activity. The amount of maltase is, however, evidently insufficient for the complete and rapid hydrolysis of the maltose.

Three preparations of mold bran from different laboratories have been tried, with no significant difference in results. Two commercial preparations of pancreatin also gave essentially equal results. Ground fresh hog

pancreas gave results similar to those with the commercial pancreatin when used in doses of roughly the same "pancreatin" content. Fresh beef pancreas, however, was much inferior in this respect.

Considerable variability in respect to the ease of breakdown was observed between different kinds of starches. Grams of corn-starch were

TABLE V

*Effect of Starch and Enzyme Concentrations on Extent of Total Sugar Production*

The quantities shown are those in 100 cc of 0.05 M acetate buffer, pH 5.4, temperature 50°

Flour or starch	Mold bran	Fresh hog pancreas	Time of digestion	Total reducing capacity	
				Wheat starch	Low protein flour (1943)
gm	gm	gm	hrs	meq	meq
2	1	1	2	2900	1940
14	3	2	4	1760	
14	3	1	4	1520	910
14	1	1	4	1280	558

TABLE VI

*Comparative Rates of Starch Digestion in Various Kinds of Flour, Meal, and Starch*

The system consisted of 23 gm of the starch or cereal product, 5 gm of mold bran, 1.7 gm of pancreas, and 0.05 gm of  $\text{CaCl}_2$  in a volume of 100 cc of 0.05 M acetate buffer, pH 5.4. It was incubated for 4 hours at 50°

Substrate	Total reducing capacity
	meq
Patent flour, low protein (1943)	910
" " high " (1942)	752
" " " " (1943)	736
Granular flour, high protein (1943)	554
Whole wheat meal (Baart, 1943)	450
Milo(kafir corn) whole meal (1943)	1290
Corn-meal (Sample A)	1710
" ( " B)	1750
Corn-starch	1480
Wheat "	1550
Potato "	645

more rapidly dissolved than those of wheat starch, while potato starch was much more resistant. The size of the starch grains does not appear to be a determining factor. The total reducing capacities found after 4 hours at 50° with several kinds of flour, meal, and starches under comparable conditions are shown in Table VI. Only the initial velocity of breakdown is indicated thereby.

## SUMMARY

Uncooked starch may be readily and completely digested by a mixture of extracts of hog pancreas and *Aspergillus oryzae* ("mold bran") The breakdown of the starch granules may be observed without difficulty under the microscope Both the pancreas and mold preparations contain factors (presumably enzymes) that are thermolabile An inorganic factor present in the ash of wheat flour is necessary for rapid digestion Calcium chloride may be used instead of this ash Approximately all of the starch is converted to sugars The products of the digestion are glucose and maltose, the former predominating The mold bran is the source of maltase

The optimum pH of the digestion is about 5.2 Temperatures up to 55° are permissible with the quantities of enzyme reported here

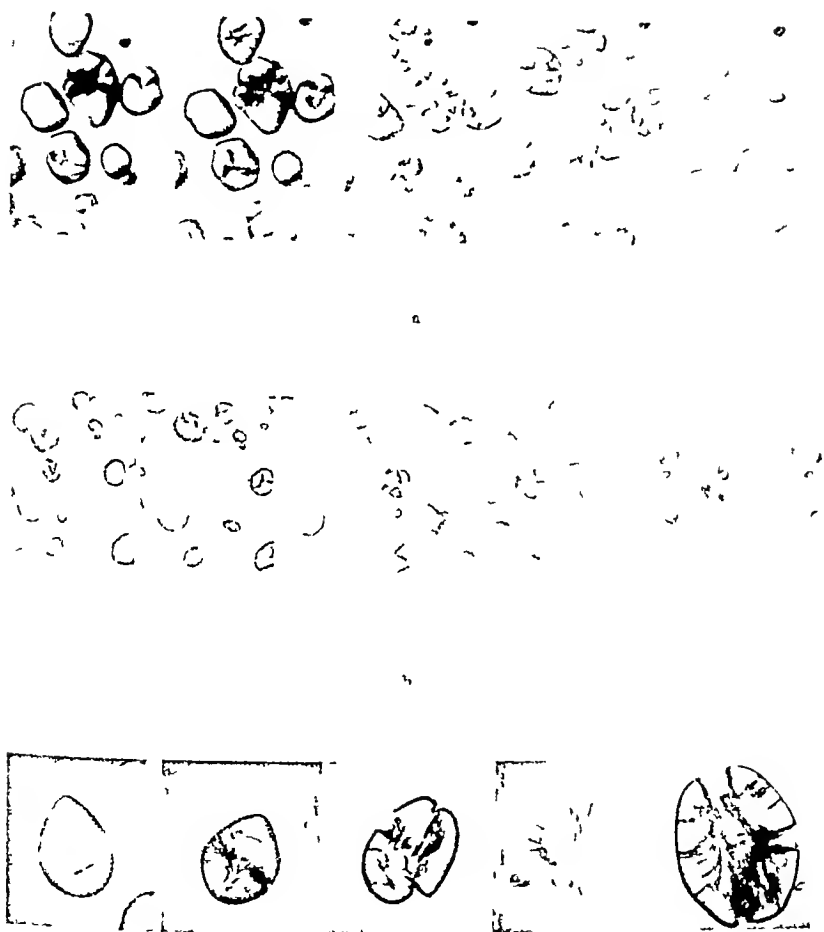
Different starches vary in the readiness with which they are broken down Potato starch is relatively resistant compared to corn and wheat starches The size of the starch grains does not seem to be a determining factor in this resistance

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## EXPLANATION OF PLATE I

FIG. 1 Starch granules at various stages of disintegration by a mold bran-pancreas mixture. Magnification ca 400. (a) Corn-starch granules after 0.2, 0.5, 3, 6, and 24 hours, (b) wheat starch granules after 0.5, 1, 2, 3, 6, and 12 hours, (c) potato starch granules after 0.5, 3, 12, and 60 hours. The last figure shows in more detail a partially disintegrated granule.



(Balls and Schwimmer Digestion of raw starch)





# THE DETERMINATION OF HISTIDINE WITH THE AID OF 3,4-DICHLOROBENZENESULFONIC ACID

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Histidine can be separated from the mixture of amino acids that results from the hydrolysis of proteins by adding an excess of a soluble salt of silver and neutralizing to pH 7.4 (1, 2), by adding an excess of mercuric chloride and neutralizing to pH 7.0 to 7.4 (3-5), or, together with the other basic amino acids, by adding an excess of phosphotungstic acid at strongly acid reaction (6, 7). Each of these precipitation methods has been employed as the first step in one or another of the various procedures that have been proposed for the quantitative determination of the proportion of histidine yielded by proteins, the object in each case being the elimination of interfering substances from the mixture of amino acids to which the specific analytical operations are applied.

The use of silver compounds in the fractionation of the basic amino acids was introduced by Hedin (6) and formed the basis of the method developed by Kossel and Kutscher in 1900 (1) for the determination of histidine, arginine, and lysine. Kossel and Patten (8) subsequently found it desirable to purify the histidine fraction not only by repeated precipitation of the silver compound, but also by precipitation with mercuric sulfate in acid solution before the histidine was adequately separated from contaminating amino acids. Under these circumstances they held that a determination of nitrogen in the fraction could be relied upon as a measure of its histidine content. Their method, with minor modifications, was extensively employed by Osborne and his associates (9) in many investigations which together laid the foundation of our present analytical knowledge of the basic amino acids of proteins.

In more recent years, the possibility of error inherent in a method that depends upon a nitrogen determination in an amino acid fraction as the final measure of the concentration with respect to the main component has led to the search for reagents which form, with histidine, salts suitable for gravimetric isolation, or which yield compounds that can be used for colorimetric estimation. Flavianic acid was advocated as a reagent for the isolation of histidine by Vickery in 1927 (10, 11) and has been widely employed (12-14), more recently Block has used nitranilic acid (15, 16). Colorimetric estimation of the histidine in such fractions has

been practiced by Csonka (17) as well as by Block (13), both of whom have used the Kapeller-Adler method (18) founded upon the bromination reaction of Knoop (19), while Hanke (20, 21) has made use of the Pauly (22) reaction with diazobenzenesulfonic acid, first placed upon a quantitative basis by Koessler and Hanke (23)

In all of these analytical investigations, with the exception of those of Hanke (21) and of Van Slyke (7), it has been found necessary to employ purification steps in addition to the initial precipitation of the histidine. Mercuric sulfate is the reagent most commonly used, and experience has shown, in those cases in which isolation has been attempted, that the previous removal of cystine by treatment of the fraction with a suitable compound of copper (24) is helpful. The procedures thus involve the repeated formation of inorganic precipitates that must be washed with great care if losses of histidine through adsorption are to be avoided or minimized, and it is clear that a reagent that can be employed for the analytical isolation of histidine directly from the initial fraction is greatly to be desired.

The observations of Doherty, Stein, and Bergmann (25) on the solubility of the salts formed by amino acids with a wide variety of aromatic sulfonic acids led to the use of 3,4-dichlorobenzenesulfonic acid as a reagent for the preparation of histidine (26) and, in turn, for its gravimetric estimation in the initial fraction obtained by precipitation with silver from proteins of high histidine content, such as the hemoglobins (27). The success that attended the use of this new reagent can be attributed to several factors. The compound of histidine with 2 moles of 3,4-dichlorobenzenesulfonic acid crystallizes unusually well and is only slightly soluble in a 10 per cent solution of the reagent. The monosulfonate on the other hand, if it can be prepared at all, must be a very soluble substance, thus there is no tendency whatever for mixtures of the two sulfonates to separate, as occasionally happens with the flavianates of histidine. The presence of a moderate quantity of hydrochloric acid in the solution does not appear to exert any unfavorable effect upon the solubility of the disulfonate, accordingly hydrochloric acid may be used as needed to liberate the histidine from the initially precipitated silver compound and to extract it quantitatively from the silver chloride. Loss by adsorption may thus be reduced to negligible proportions. Furthermore, inasmuch as the crystallization of the histidine salt takes place in a strongly acid solution, not only the histidine itself but any other contaminating amino acids are completely ionized as cations, and effects upon the solubility due to the presence of dipolar ions (28) are eliminated. Finally, cystine, which appears to be the most commonly present contaminant of histidine fractions obtained from protein hydrolysates by precipitation with silver at pH 7.4, forms a far more soluble salt with the reagent than does histidine.

and only a trace is thus to be anticipated as an impurity in the crystals of the histidine compound. Any such impurity is readily eliminated by recrystallization.

The development of a procedure that can be applied to the determination of histidine in proteins of moderately low histidine content accordingly seemed feasible and such a procedure is described in the present paper together with data that illustrate its application to a few common proteins.

#### EXPERIMENTAL

*Preparation of Hydrolysate*—The quantity of protein to be taken depends upon the number of replicate analyses contemplated. About 5 gm per single determination are satisfactory in the case of a protein that yields from 1 to 3 per cent of histidine, for the analyses described below approximately 25 gm of air-dry material were employed and the determinations were carried out in quadruplicate.

The sample of protein is heated on the steam bath with about 700 ml of 20 per cent hydrochloric acid until all danger of frothing is past, and is then boiled for 24 hours on a hot-plate under a reflux condenser. The solution is repeatedly concentrated *in vacuo* to remove excess of acid, diluted to 250 ml, and 1 ml aliquots are removed for determinations of nitrogen, from which the exact quantity of protein present is computed.<sup>1</sup> The remainder, including rinsings of the pipettes, is diluted and boiled with the minimal quantity of norit needed to decolorize it, usually about 3 gm. The norit is washed by being boiled with water three times successively after removal from the filter, and the solution, together with the washings of the norit, is concentrated and made to 250 ml. Of this, four 50 ml aliquots are taken for the histidine analyses.

*Preparation of Histidine Fraction*—The precipitation of the histidine is conveniently carried out in a 500 ml centrifuge bottle. A 20 per cent solution of silver nitrate is added from a burette with continuous stirring until the presence of excess (brown precipitate) can be demonstrated by testing a drop of the solution on a watch-glass with alkali. About 70 ml are usually required. The test sample is washed back, and the solution is then carefully neutralized to pH 7.4 by adding 1 N sodium hydroxide with continuous stirring. This is conveniently done by transferring the alkali from a graduated cylinder with a fine tipped 10 ml pipette, while the bottle is rotated by hand. The object of proceeding in this way is to avoid the production of local alkalinity and the consequent precipitation of arginine. The amount of alkali required usually somewhat exceeds

<sup>1</sup> It is customary in this laboratory to conduct macro-Kjeldahl analyses in quadruplicate, the digestion time being 8 hours for proteins or for samples that contain histidine or lysine. For a discussion of the precautions necessary when trustworthy values for protein nitrogen are required see Chibnall, Rees, and Williams (29).

the volume of silver nitrate solution previously added. As the end-point is approached, the white precipitate flocculates and settles readily so that a few ml of clear solution can be removed with a pipette and tested with bromothymol blue indicator solution. Comparison is made with a buffer solution at pH 7.4 similarly colored and the test samples are returned quantitatively to the main solution. The volume at this stage is usually about 350 ml, owing to the liberal use of wash water to rinse the precipitate from the walls of the bottle before it is allowed to settle. The precipitation and adjustment of the reaction in each of the replicates must be carried out with the same care.

The precipitate is centrifuged and the supernatant solution is decanted through a thin layer of paper pulp on a 4 inch Hirsch funnel in order to retain any precipitate that may have been disturbed. Washing is carried out by loosening the precipitate with a rod and then shaking it with about 300 ml of water in the stoppered bottle. The froth produced during the first washing usually causes a small amount of precipitate to float after centrifugation, but this is retained on the filter. The precipitate is washed three times, but it is wise to set aside the filtrate after the second washing, since the third occasionally becomes colloidal and may need to be poured through the filter repeatedly before it becomes perfectly clear. By this thorough washing, most of the nitrate ion is removed as well as any sparingly soluble silver salts, such as those of glutamic and aspartic acids, which may have been precipitated.

The precipitate is again suspended in water and 3 ml of concentrated hydrochloric acid are added, after which the bottle is stoppered and thoroughly shaken. The silver chloride is centrifuged and the clear solution is decanted through the same Hirsch funnel into a clean filter flask, care being taken that all solid particles on the paper pulp are moistened with the acid solution. If the filtrate is not perfectly clear, it is refiltered. The silver chloride is washed three times successively with about 250 ml of water that contains a single drop of hydrochloric acid, the last washing being carried out with hot water, all washings are decanted through the same filter.

The acid extract of the precipitate contains the histidine derived from the protein together with minor quantities of other substances that form insoluble silver compounds under the conditions. Of these cystine and cysteine have been identified by color tests as components, the cysteine presumably having been precipitated as the silver mercaptide (30)<sup>2</sup>

<sup>2</sup> Only in the analysis of human hair was the quantity of cysteine present significant. In this case, rough colorimetric tests indicated that cystine and cysteine were present in the acid solution immediately after liberation from the silver compounds in the proportion of 3:1.

The extract together with the washings is concentrated *in vacuo* in a 3 liter flask to a volume of a few ml but not to dryness, owing to the possibility of oxidation of the histidine by the trace of nitric acid that is invariably present. The solution is washed quantitatively through a thick pad of paper pulp in a 2 inch Hirsch funnel to remove any trace of silver chloride, the filtrate being received in a 500 ml flask in which it is again concentrated *in vacuo* to small volume<sup>3</sup>. The concentrated solution is then quantitatively transferred with the aid of a little hot water to a 50 ml beaker. The final volume should be brought to 20 ml (calibration mark on the beaker) by evaporation on a steam bath if necessary.

*Isolation of Histidine Bis-3,4-dichlorobenzenesulfonate*—The crystallization of the disulfonate is best allowed to take place in the presence of a concentration of the reagent of about 10 per cent<sup>4</sup>. This is provided if 3 gm of the dihydrate of 3,4-dichlorobenzenesulfonic acid are added in dry form. The solution is warmed until all precipitated histidine salt is in solution, is allowed to stand at room temperature for a few hours until crystallization is well advanced, and is then placed in the refrigerator. Complete equilibrium appears to be approached somewhat slowly, and, in the analyses discussed below, the solutions have been allowed to stand at low temperature, occasionally being stirred, for at least 3 days before being filtered. Filtration is accomplished on weighed sintered glass crucibles of medium porosity, the mother liquor being received in a small beaker placed in the filtration apparatus and used as required to complete the transfer of the crystals to the crucible. The operation should be carried out in a cold room, if one is available, but can be done effectively at room temperature if the apparatus is chilled and the transfer is made rapidly. After the mother liquor has been sucked from the crystals, they are washed with from 3 to 5 ml of a chilled 4 per cent solution of the reagent and the crucibles are wiped and dried for a few hours in a vacuum desiccator over sulfuric acid. To remove the residual traces of reagent, the crystals are washed with 30 ml of ether used in three equal portions and allowed to run through slowly, care being taken that all of the internal surface of the crucible is moistened with ether. The crucibles are then dried at 105° for a short time, cooled in a desiccator, and weighed. Even when completely dry the substance is not notably hygroscopic. The weight of

<sup>3</sup> The special distillation apparatus described some years ago (31) is convenient for the concentrations, since it permits the interchangeable use of ring neck flasks of different sizes. The filtrations are carried out with the use of a dome-covered Pyrex distillation apparatus (Central Scientific Company, catalogue No 12910) fitted with funnel and suction line mounted in a 2-hole stopper.

<sup>4</sup> No significant difference has been detected in the solubility in a 10 and in a 15 per cent solution of the reagent.

the disulfonate is converted to the weight of histidine by multiplication by the factor 0.2548

In order to be assured of the purity of the histidine salt, recrystallization is necessary. This is accomplished by transferring the greater part of the crystals with a spatula to a 50 ml beaker, which is then placed in the filtration apparatus, and the residual salt in the crucible is washed through quantitatively into it with a little boiling water. The volume of the washings and rinsings of the apparatus is kept below 20 ml. After the solution is diluted to 20 ml, 2 gm of the reagent are added and the beaker is warmed until the crystals are dissolved. Crystallization is effected as already described. Equilibrium is apparently reached fairly promptly, although in the analyses described below 1 day, or more, was allowed. The loss of weight on recrystallization has been found to vary from 10 mg to about 50 mg, depending on the purity of the original product. Accordingly a second recrystallization has in most cases been carried out. The average loss observed during the second recrystallization in thirty seven unselected cases was  $11.1 \pm 1.7$  mg, while the average loss in a smaller group of especially fine preparations was 10.2 mg. This figure remained essentially constant when further recrystallizations were carried out and accordingly has been used in correcting the weight of the samples of recrystallized salt for the solubility under the specified conditions employed.

For identification, the decomposition point of the product from each aliquot is taken and nitrogen is determined in a pooled sample. The constancy of the result obtained after successive recrystallizations and correction for solubility furnishes final proof of the purity of the histidine compound isolated.

The value assigned to the proportion of histidine yielded by the protein under investigation should manifestly be calculated from the weight of the compound after correction for the solubility in each of the mother liquors from which it has been separated. An additional correction of 10.2 mg has accordingly been applied to the weight of the recrystallized preparations to allow for the solubility loss in the original mother liquor.

Although none of the operations presents any particular difficulty, experience has shown that somewhat subtle differences in personal technique may at times influence the final result. Until some experience has been gained with the initial precipitation, tests for the presence of arginine in the mother liquor of the first crop of disulfonate should be made by adding a little flavianic acid to a sample. If the characteristic orange plates of arginine flavianate separate within 24 hours, the result for histidine should be viewed with suspicion and special care taken with the recrystallizations. Aside from this, the most likely points at which errors may

arise are in the transfers of the solutions and in the concentrations. All transfers are carried out with careful draining of the apparatus and with repeated washings, the color reaction with diazobenzenesulfonic acid being employed to demonstrate completion of the operation. From four to six rinsings are usually sufficient and a definite technique is readily established and is subsequently rigidly maintained. Aberrant individual results can sometimes be traced to loss by entrainment during the vacuum distillation. This step must therefore be carried out with careful attention.

Loss of histidine through oxidation, owing to the presence of a trace of nitrate ion in the histidine fraction, must be regarded as a possibility during the later phases of the concentrations, but experience shows that oxidation losses usually affect the contaminants, (presumably cystine) far more than they do the histidine. An illustration is provided by the last analysis shown in Table I. The histidine fraction from this aliquot was inadvertently allowed to evaporate to dryness during the preliminary concentration. The weight of the original crop of crystals of the compound was significantly affected, but the weight of the purified material was too little diminished to warrant exclusion from the average although it gave the lowest observation in the group.

*Recovery of Known Amounts of Histidine*—The following experiments indicate that the over-all losses can be kept within reasonably satisfactory limits under the working conditions adopted. Quadruplicate samples of 0.1148 gm. of histidine, weighed as the monohydrochloride monohydrate, were recovered by direct crystallization of the disulfonate from a solution of the reagent to the extent of  $98.3 \pm 0.1$  per cent without correction for solubility, or  $99.4 \pm 0.1$  per cent when corrected. Duplicate samples of 0.3651 gm. in the same way gave 99.4 and 99.3 per cent without correction, 100.0 and 99.9 per cent when the solubility correction was applied. A similar pair of samples was carried through the entire procedure of precipitation with silver and isolation as the disulfonate with recoveries of 98.3 and 97.8 per cent without correction, 98.9 and 98.5 per cent corrected.

In another series of tests, the individual filtrates from the precipitates of histidine silver obtained in the course of an analysis of a protein were treated with an excess of hydrochloric acid, filtered, and concentrated, and a known quantity of histidine as the monohydrochloride was added to each. The recovery of the added histidine in this case was therefore carried out under exactly the conditions encountered during an analysis with the exception that the solution contained a little additional sodium nitrate and was somewhat deficient in cystine. A set of filtrates from an analysis of casein (4.799 gm. per aliquot) to each of which 0.1027 gm. of histidine had been added gave an average recovery of  $102.8 \pm 1.5$  per cent after the samples of the histidine compound had been recrystallized.



twice and corrected for solubility for each of the three crystallizations. A similar set of filtrates from the analysis of aliquots that contained the amino acids derived from 5 702 gm of edestin and to which 0 1204 gm of histidine had been added gave an average recovery of  $101.3 \pm 0.2$  per cent, again after two recrystallizations and correction.

To each of a set of hydrolysates that represented 4 719 gm of edestin per aliquot, 0 2000 gm of histidine was added and the histidine was isolated

TABLE I  
*Histidine of Edestin*

The weights of recrystallized disulfonate were corrected by adding 10.2 mg per 20 ml of mother liquor

Quantity of protein taken (1)	Yield calculated from weight of bis 3,4-dichlorobenzenesulfonate				Decomposition point of disulfonate (6)	N content of disulfonate theory 6.90 per cent (7)
	Initial separation uncorrected for solubility (2)	Recrystallized once corrected for solubility (3)	Recrystallized twice corrected for solubility (4)	Recrystallized twice corrected for solubility of initial crystals (5)		
gm	per cent	per cent	per cent	per cent	C	per cent
4 798	2.67	2.64	2.53	2.59	278-279	6.89
	2.61	2.49	2.42	2.47	278-279	6.92
	2.51	2.49	2.49	2.54	278-279	6.82
	2.67	2.65	2.62	2.68	278-279	6.93
4 695	2.44	2.45	2.41	2.47	278	7.01
	2.63	2.53	2.44	2.50	279	6.96
5 702	2.69	2.52	2.53	2.53*	279-280	
	2.65	2.47	2.48	2.52*	281	
	2.68	2.48	2.48	2.52*	279.5-280	
	2.44	2.40	2.39	2.43*	281	6.83†
Average	2.59	2.51	2.48	$2.52 \pm 0.07$		6.91

\* This set was recrystallized three times in order to demonstrate that constant solubility had been attained. The average loss in the second crystallization was 0.0098 gm, in the third 0.0111 gm. These figures were computed after addition of 40.8 mg to the weights of the three times recrystallized specimens.

† Nitrogen determined in pooled sample

After correction for one crystallization and one recrystallization and for the 2.52 per cent of histidine yielded by the protein (see Table II), the recovery was  $99.9 \pm 2.8$  per cent. In a similar experiment carried out with zein (4.428 gm), the recovery of 0.1124 gm of histidine, after correction for solubility and for the 0.85 per cent of histidine derived from the zein (see Table II), was  $96.6 \pm 1.8$  per cent. This last experiment represents what is probably a lower limit beyond which satisfactory results can scarcely be expected without special modification of the technique,

inasmuch as the correction for the histidine contributed by the zein (about 37 mg) is known with a precision no greater than 6 per cent (see Table II)

It would seem from the foregoing that an accuracy of an order of somewhat better than  $\pm 3$  per cent is to be anticipated with samples of proteins that yield 100 mg or more of histidine

*Histidine of Proteins*—Because of its importance as a standard of comparison in many chemical and physical investigations, considerable attention has been given to the determination of histidine in edestin. The preparation used was made in this laboratory and had been previously analyzed both for arginine (32) and for histidine (27). Complete data for the recrystallizations of the histidine salt obtained from each aliquot in three separate analyses are shown in Table I to illustrate the reproducibility of the individual determinations as well as the method of calculation of the results. Column 2 shows the proportion of histidine calculated from the weight of the crystals that separated from the initial histidine fraction, without correction for solubility. Columns 3 and 4 show the results of two successive recrystallizations of this material, corrections of 10.2 mg of disulfonate in each 20 ml of 10 per cent solution of the reagent used as a mother liquor having been applied. The differences between the figures in Columns 2 and 3 show the effect of the removal of the small proportion of impurity present in the first crop of crystals, while the constancy of the result during subsequent recrystallizations furnishes a demonstration of the adequacy of the purification. The third analysis, carried out after considerable experience with the method had been obtained, is particularly satisfactory.

A correction for the solubility of the initial crop of crystals is applied in Column 5 to obtain the most probable value for the final result of the analysis. In spite of a partial, and occasionally a complete, compensation of errors, it is clear that the uncorrected weight of the initial crop cannot be relied upon as an accurate measure of the histidine yielded by the protein.

The present value for the histidine yielded by edestin confirms moderately closely the two results, 2.41 and 2.39 per cent, that have appeared recently from Chibnall's laboratory (33, 34) and which were obtained from the weight of the isolated disulfonate. The present data are also in agreement with the result of an earlier analysis by a less fully developed form of the procedure (27) in which six determinations, the results of which were calculated from the uncorrected weight of the initial crystalline material, gave an average of  $2.64 \pm 0.05$  per cent. This figure corresponds with the average of  $2.59 \pm 0.10$  per cent for the histidine calculated similarly from the present data. The earlier analysis was recognized to be a little too high since, although the material weighed was pure as far as could be

told from the decomposition points and nitrogen content, it was dark in color and was doubtless slightly contaminated

Table II shows the average results of the analysis of a number of proteins. The quantity of protein represented by the individual aliquots analyzed

TABLE II  
*Histidine of Proteins*

(1)	N content of protein preparation corrected for ash and moisture (2)	No of determinations (3)	Yield calculated from weight of bis-3 4-dichlorobenzenesulfonate				Histidine N of protein N (8)	N content* of histidine salt theory 6.90 per cent (9)
			Initial separation uncorrected for solubility (4)	Recrystallized once corrected for solubility (5)	Recrystallized twice corrected for solubility (6)	Recrystallized corrected for solubility of initial crystals (7)		
	per cent		per cent	per cent	per cent	per cent	per cent	per cent
Serum albumin (human)	16.45	4	3.27	3.18	3.16	3.22 ± 0.03	5.30	6.90
Serum albumin (bovine)	16.22	7	3.38	3.32	3.30	3.35 ± 0.11	5.59	6.89
Fibrin (human)	16.89	4	2.34	2.33		2.39 ± 0.02	3.83	6.94
" (bovine)	16.77	4	2.05	2.03	2.01	2.06 ± 0.08	3.33	6.93
Edestin	18.69	12	2.59	2.51	2.48	2.52 ± 0.07	3.65	6.89
Pumpkin seed globulin	18.55	4	2.06	2.06		2.12 ± 0.02	3.19	6.92
Watermelon seed globulin	18.64	4	2.37	2.34	2.32	2.37 ± 0.02	3.45	6.83
Soy bean globulin A	17.04	4	2.06	2.03	2.03	2.08 ± 0.02	3.31	6.97
Soy bean globulin B	16.93	4	2.04	2.02		2.08 ± 0.04	3.33	6.94
Arabin	18.29	4	2.28	2.26	2.26	2.31 ± 0.03	3.42	6.85
Casein	15.75	8	2.56	2.49		2.53 ± 0.09	4.35	6.90
Gliadin (wheat)	17.66	4	1.76	1.74	1.73	1.79 ± 0.02	2.75	6.96
Zein	16.13	4	0.86	0.79	0.80	0.85 ± 0.05	1.43	6.90
Gelatin	18.27	4	0.45	0.43	0.45	0.51 ± 0.04	0.76	
Hair (human)	16.93	4	0.72	0.73		0.77 ± 0.03	1.23	6.85

\* The decomposition points of the individual preparations have not been reported, since all were satisfactory. Of 95 preparations, one decomposed at 275-276°, and five at 276-277°. Of the rest, all save two decomposed over ranges of from 1-1.5° within the limits 277-282°. These two preparations decomposed at 282-283° but were doubtless heated too rapidly.

was in most cases between 45 and 50 gm so that, with the exceptions of zein, gelatin, and human hair, the quantity of histidine isolated was 100 mg or more. The human serum albumin and fibrin, as well as the bovine serum albumin, were prepared in the laboratory of

Professor Cohn at the Harvard Medical School,<sup>5</sup> the other preparations, with the exception of the arachin, were made in this laboratory and most of them have been described previously (32). The pumpkin seed globulin was obtained from seeds of *Cucurbita moschata*, variety striped cushaw, and the watermelon seed globulin from seeds of *Citrullus vulgaris*, variety Halbert honey, respectively, in 1941 and in 1940 by the methods described by Vickery, Smith, Hubbell, and Nolan (35). The soy bean globulin 'A' was prepared in 1915 and represents the more soluble fraction of the total bean globulins. The product had been obtained by precipitation with ammonium sulfate, after removal of a less soluble fraction, between the limits 0.6 and complete saturation at an unspecified pH, and had been subsequently redissolved and dialyzed. The variety of beans used was unfortunately not recorded. The soy bean globulin B was recently prepared by Dr G. W. Pucher and Mr L. S. Nolan of this laboratory from fat-extracted meal from beans of the variety Illinois. The proteins were extracted with saturated sodium chloride solution at pH 7.1, and the extract was completely clarified with a Sharples supercentrifuge and dialyzed for 24 hours, at which time the salt concentration was 0.07 M. The material that separated after adjustment to pH 5.3 was redissolved in 1.5 M sodium chloride at pH 8.0, and the solution was again clarified and dialyzed, and was then adjusted to pH 5.0. The product was dehydrated with acetone and equilibrated with respect to moisture content with laboratory air. It presumably represents the less soluble globulins of the bean and corresponds more or less, at least with respect to the general method of preparation, to the soy bean protein defined by Osborne and Campbell (36) as glycinin, although the material, like the other preparation analyzed, is lower in nitrogen content. In the course of a very careful study of the proteins obtained from this variety of soy beans, it has not been found possible to duplicate the nitrogen content of 17.53 per cent observed by those investigators.

A careful redetermination of the nitrogen content of the preparation of casein analyzed led to a slightly higher result than was previously obtained (32) but one which agrees with and confirms the value of Chibnall, Rees, and Williams (29).

<sup>5</sup> The products of plasma fractionation employed in this work were developed by the Department of Physical Chemistry, Harvard Medical School, Boston, Massachusetts, under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Harvard University. The fractions of bovine plasma were prepared at the Armour Laboratories, Chicago, Illinois, under a contract, recommended by the Committee on Medical Research between the Office of Scientific Research and Development and the Armour Laboratories. Grateful acknowledgment is made to these agencies.

## DISCUSSION

The most probable final values obtained in the present determinations of histidine in proteins are given in Column 7 of Table II. On the assumption that the data from the recovery experiments are applicable, the figures are probably within 2 to 3 per cent of the true proportion of histidine yielded by these proteins. The results with analytical methods for the determination of amino acids that depend upon isolation are commonly assumed to be in error through failure to bring about complete separation of the substance. This assumption is doubtless justified in most cases, since suitable correction for the solubility of the isolated compound is only infrequently possible, and many instances are to be found in the literature of histidine analysis (see Table III). In the present method, reliance is placed in the first place upon the complete insolubility of the histidine compound of silver that is formed at the isoelectric point of histidine. No evidence has come to the attention of the writers that suggests that this substance possesses a solubility that is significant in experiments on the scale of these. Even the most careful examination of filtrates from precipitates of histidine silver for the presence of amino acid nitrogen would not provide convincing evidence unless preparations of histidine of a degree of purity that has only very rarely been attained were employed. Furthermore such experiments would beg the real question, since what is wanted is the solubility in a solution of mixed amino acids at a hydrogen ion activity at which the concentration of dipolar ions approaches its maximum. The experimental problem involved is thus one of considerable complexity and is beyond the scope of the present study.

The solubility of the dichlorobenzenesulfonate under the experimental conditions employed is, however, easy to estimate with an accuracy adequate for practical purposes. The measure that has been adopted is the actual loss of weight experienced in the recrystallization of purified samples of the substance, a quantity that has been found to be substantially constant. Inspection of Columns 5 and 6 of Table II shows that the application of the correction in most cases suffices to bring the average weight of the product after two recrystallizations into complete agreement with the weight after only one. This has seemed ample justification for employing the same correction for the solubility of the salt in its original mother liquor despite the fact that this mother liquor is usually somewhat colored and is known to contain a moderate amount of hydrochloric acid, a little cystine, and possibly traces of other substances. The presence of 10 per cent or more of the reagent itself can probably be relied upon to minimize any solubility effects arising from these other components. The standard deviation of the individual determinations from the mean

has been computed for each set of analyses. This quantity is small in those cases in which a single hydrolysate was analyzed in quadruplicate, indicating a satisfactory degree of reproducibility within a set. It is appreciably larger, however, when the final figure is the average of two or more sets and amounts to a little over 3 per cent of the quantity measured in the bovine serum albumin and to a little under 3 per cent for the casein and the edestin analyses. Sufficient experience has been had with the analysis of edestin to indicate that this figure is a reasonably valid estimate of the precision that is to be expected.

The small standard deviation is not to be taken as evidence of the accuracy of the determinations. The question whether the histidine of human serum albumin does or does not differ from that of bovine serum albumin is not conclusively answered although there is some likelihood that a small difference exists. More extensive analyses of these and of other preparations of the respective proteins would be required to establish the relationship.

The results with zein and gelatin warrant mention in view of the obvious fact that the proportion of histidine yielded by these proteins is so small as to render quantitative isolation from as little as 5 gm somewhat difficult. The reproducibility is far less satisfactory than in the other analyses. These are proteins with which a material increase in the size of the sample taken would be desirable. Human hair was analyzed as an example of the application of the method in the most unfavorable case, that of a protein low in histidine and high in cystine. No special difficulties were encountered.

A comparison with values from the literature is shown in Table III, the data being segregated according to the method of analysis employed. The various forms of the fundamental Kossel and Kutscher procedure all, like the present method, rely upon preliminary precipitation of histidine with silver. They differ with respect to the purification steps and the means employed for the final measurement. The colorimetric method of Hanke and Koessler was originally applied to the base fractions obtained with phosphotungstic acid, but Hanke subsequently secured more satisfactory results by applying it to histidine fractions precipitated with silver. It is these data that are shown in Column 5 of Table III.

Van Slyke's method depends on precipitation with phosphotungstic acid and subsequent calculation from the quantities of amino, non-amino, arginine, and cystine nitrogen in the precipitate. Among the other methods that have been proposed are those of Lang (37), who precipitated the histidine at neutral reaction with mercuric chloride, dissolved the precipitate in cyanide, and made photometric measurements essentially according to Koessler and Hanke's (23) method, of Albanese (38), who separated

easy, but the present tendency in amino acid analysis is toward the development of methods that yield many replicate results within a short time and which require only small quantities of protein. The purpose is to provide an isolation technique that is as reliable as possible in order to obtain data with which even simpler methods can be controlled.

#### SUPPLEMENTARY OBSERVATIONS

*Preparation of 3,4-Dichlorobenzenesulfonic Acid*—The method for the preparation of this sulfonic acid (26) can be greatly shortened if the mixture of sulfuric acid and *o*-dichlorobenzene is stirred rapidly while being heated at 170–180°. If the operation is carried out under a hood, no reflux condenser is necessary and sulfonation is practically complete in about 3 hours. The common test for the completion of sulfonation, namely the failure to form a turbidity when a drop of the sulfonation mixture is diluted with water, is not reliable, owing to the presence of a small proportion of *p*-dichlorobenzene in the commercial *o*-dichlorobenzene that has been used. This isomer is not sulfonated under the conditions. Yields of 80 per cent of sulfonic acid dihydrate have been regularly obtained without any attempt to separate second crops by concentration of the mother liquor. An additional 10 per cent or more can be readily secured if this is done.

A trace of impurity insoluble in concentrated aqueous solutions of reagent frequently noted in earlier preparations has been identified as the calcium salt of the sulfonic acid, the calcium being presumably derived from the norit employed for the decolorization. Acid-extracted norit should therefore be used for this operation.

*Cystine Bis-3,4-dichlorobenzenesulfonate*—A mixture of 1 gm of cystine and 4 gm (nearly 4 moles) of dichlorobenzenesulfonic acid dihydrate was warmed with 12 ml of water, when all passed into solution. After being chilled overnight, the solution was evaporated to 8 ml, as no crystallization had occurred. Crystals of the salt then separated in the form of thick plates of regular rhombic outline, sometimes several mm in diameter, and frequently aggregated into piles. After being filtered, dried *in vacuo*, and washed with ether, they weighed 1.45 gm (50 per cent yield). The mother liquor was evaporated successively to 3 and to 1 ml and two additional small crops were recovered, amounting in all to a yield of 91 per cent. The substance decomposes with darkening and gas evolution at 215°. When stirred with twice its weight of water, it passes into solution but is at once hydrolyzed and deposits free cystine. The crystals are soluble at room temperature in 3 times their weight of a 4 per cent solution of the sulfonic acid and this solution deposits crystals when chilled only after a little evaporation has occurred. It is thus difficult to account for

the presence of cystine in the initial crop of histidine disulfonate obtained in the course of the analysis of a protein on the grounds of the solubility of the cystine salt. The substance gives the characteristic color tests for cystine. The nitrogen content of the specimen obtained was 3.97 per cent, theory 4.03 per cent.

*Cysteine 3,4-Dichlorobenzenesulfonate*—1 gm. of cystine was mixed with 25 ml. of 4 N hydrochloric acid and a little finely granulated tin. After standing overnight, the solution was filtered, treated with hydrogen sulfide, filtered, and evaporated *in vacuo* to a sirup. This was treated with 3 gm. of dichlorobenzenesulfonic acid and diluted to 10 ml., warmed, and then chilled overnight. Crystals separated as a coherent mass in the form of bunches of heavily striated coarse needles or prisms. Individual crystals that separated from the mother liquor during examination under the microscope were in the form of considerably elongated hexagons that could be described as flat prisms with acuminate ends. Nitrogen content 4.13 per cent, theory 4.02 per cent. The salt is freely soluble in twice its weight of 4 per cent sulfonic acid solution at room temperature. It melts with slight darkening and the evolution of a little gas to a clear, slightly brownish oil at 197° when slowly heated. The oil solidifies after being cooled and, when again heated, melts at about 187° with further decomposition and gas evolution. Although clearly less stable, the salt thus resembles in behavior the dichlorobenzenesulfonates of leucine and phenylalanine (26) which possess true melting points at about the same temperature.

The properties of these salts of cystine and cysteine are accordingly such that quantitative separation from the histidine salt by recrystallization, should one or both be present as contaminants, is entirely feasible.

#### SUMMARY

Histidine is quantitatively separated from the mixture of amino acids produced by the hydrolysis of proteins by precipitation as its silver compound, and is isolated as the crystalline bis-3,4-dichlorobenzenesulfonate without further treatment of the histidine fraction. This substance is purified by recrystallization. Losses from adsorption upon inorganic precipitates are thus minimized, while losses due to the solubility of the salt are compensated by correction by a quantity equal to the constant loss in weight observed after repeated recrystallization of the dichlorobenzenesulfonate under the experimental conditions adopted. The purity of the isolated compound is established by the decomposition point, the nitrogen content, and the constancy of its solubility.

Recovery of known amounts of histidine under experimental conditions that closely resemble those encountered in actual analytical operations



is in general within 3 per cent of quantitative. Except with proteins of very low histidine content, the reproducibility of individual determinations is between 2 and 3 per cent.

Analyses of a number of proteins gave results that confirm remarkably closely the early data obtained by Kossel and by Osborne and their associates, who employed the long and difficult Kossel and Patten procedure, and also much of the data of Hanke who employed a colorimetric method based upon the Pauly reaction.

The failure to confirm a number of carefully made determinations of histidine in proteins, in cases in which use was made of one or another of the modifications of the Kossel procedure that have been proposed in recent years, suggests that these modifications have not in fact resulted in improvement in accuracy. Whether the low results to which they lead arise from losses due to the extensive use of inorganic precipitants, or to the inadequacy of flavianic acid as a reagent for the quantitative isolation of histidine, is not certain, but it is clear that flavianic acid should now be discarded for this purpose.

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# THE DETERMINATION OF TOTAL BASE OF SERUM BY ION EXCHANGE REACTIONS OF SYNTHETIC RESINS

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Measurement of total base of blood serum supplies information indispensable for the complete description of the electrolyte balance in many physiological and pathological states. Older methods involving separate analyses of the principal cations or their conversion to sulfates, with subsequent determination of sulfate, were time-consuming, while the latter was of doubtful accuracy. More recently, electrodialysis, introduced by Keys (1), has made possible analyses of improved accuracy and speed. It has been our experience, however, as well as that of others, that electrodialysis did not always yield dependable figures for total base unless continued for 12 to 18 hours at relatively low current densities and Consolazio and Talbott (2) have questioned its accuracy when applied to the serum of certain hospitalized patients. Sunderman (3) has proposed the measurement of conductivity for determination of the total base of serum. His method requires the use of a statistically determined conversion factor from which individual deviations in some instances may cause significant error.

The high efficiency with which adsorbents function in ion exchange appeared to offer the basis of a simple and rapid method for measurement of the total base of serum that would also avoid objectionable features of the existing methods. The action of synthetic resins as exchange adsorbents has already been characterized (4). Ion exchange was shown to be stoichiometric in nature and the titration of the effluent from a hydrogen exchange column was exactly equivalent to the salt concentration in the solution being treated. Two types were available. One, a cation exchanger which acts by the adsorption of the cation on an insoluble resin "lattice," with simultaneous release of hydrogen ions, is of the type formed by the condensation of aromatic hydroxysulfonic acids with formaldehyde. The other is an acid-adsorbing resin of the alkylene polyamine type. The latter splits easily hydrolyzable salts such as ammonium or ferric chlorides, but does not react appreciably with neutral salts such as sodium chloride. Its action has been thought to be that of adsorption on the resin framework of the acid formed by hydrolysis of the salt. An attempt was made to use the acid-adsorbing resin (Amberlite IR-4) by combining simultaneous

electrolysis and adsorption, the anode being surrounded by a nylon bag containing the resin. Results indicated that neutral salts were split with resulting anion adsorption on the resin. Cations remained in the solution and could then be titrated in the cathodic cup. This method might have enabled accurate measurement of the total base of serum but appeared to offer little advantage, in so far as simplicity was concerned, over the electro-dialysis method of Keys as amplified by Consolazio and Talbott. The alternative procedure, the adsorption of base by means of a cation-adsorbing resin and titration of the effluent acid, functioned so efficiently in preliminary trials with serum that it was decided to investigate and develop this reaction.

A cation-adsorbing resin (Amberlite IR-100) was converted to the hydrogen form by treatment with dilute acid. Upon contact with the cations in serum an exchange occurred in which hydrogen ions from the resin were liberated in proportion to the cations taken up from the serum. The resulting solution was distinctly acid and contained chloride, bicarbonate, phosphate, protein, etc., in the form of acid anions. The bicarbonate ion could not be determined with the other anions. To overcome this difficulty, carbon dioxide was removed by passing a stream of carbon dioxide-free air through the acid solution. The solution so treated was then titrated with standard base to the pH of a control sample of serum similarly aerated but not subjected to treatment with the resin. Base combined as bicarbonate was obtained from the carbon dioxide-combining capacity determined separately.

#### *Apparatus and Reagents—*

**Adsorption tube.** A glass tube, measuring approximately 70 mm in length and 8 mm in diameter, with a cup 40 mm long and 20 mm in diameter at the top and a capillary tube of 2 mm bore at the bottom.

**Micro burette** calibrated in 0.01 ml intervals, or Rehberg micro burette. A Krogh-Keys pipette has proved to be advantageous for measurement of 0.2 ml samples of serum and standard.

**Cation-adsorbing resin.** Amberlite resin IR-100<sup>1</sup> is activated by shaking with 4 per cent hydrochloric acid (approximately 2 ml to 1 gm of resin) until "color throwing" ceases. The resin is then washed with distilled water until the filtrate is neutral, dried in air on a Buchner funnel, and stored until used. Its activity is tested before each batch of newly activated

<sup>1</sup> Amberlite IR-100 is now marketed in purified form already converted to the hydrogen derivative as "Amberlite IR-100-H analytical grade." It is obtainable from the Resinous Products and Chemical Company, Washington Square, Philadelphia.

We are greatly indebted to the manufacturers for supplies of the synthetic resins used in these studies.

resin is used The activity of the resin is tested by adding 0.2 ml of the standard sodium chloride solution and washing it through the resin with two 5 ml portions of distilled water The effluent is then titrated with the standard sodium hydroxide, 1 drop of indicator being used Generally this standard salt determination will have values from 0 to 4 milliequivalents per liter high, depending on the efficiency of the activation and of the washing procedure If the value is lower than the expected value by more than 3 milliequivalents per liter, the resin in the tube should be reactivated by washing with 10 ml of 4 per cent hydrochloric acid and then with water to neutrality <sup>2</sup>

Caprylic alcohol

Sodium hydroxide 0.2 M if the Rehberg micro burette is used, or 0.02 M if the 0.01 ml interval burette is used Protect from uptake of carbon dioxide

Standard sodium chloride, 0.150 M

Phenol red, 0.04 per cent Brom-thymol blue, 0.04 per cent, may be substituted advantageously when the carbon dioxide-combining capacity is low

Hydrochloric acid, approximately 4 per cent by weight 9 ml of "concentrated" hydrochloric acid diluted to 100 ml

Carbon dioxide-free air is obtained by passing the stream of air through a wash bottle containing approximately 30 per cent sodium hydroxide solution It is introduced into the solution by means of a capillary with a fine tip

### Procedure

*Preparation of Adsorption Tube*—Approximately 2 gm of the dry activated resin are placed in an adsorption tube whose outlet is loosely plugged with Pyrex glass wool to prevent resin from falling through The tube is suspended in a test-tube rack over a test-tube of approximately 20 mm diameter and 150 mm length if a Rehberg micro burette is used, or over a 30 ml beaker if an ordinary burette is used

*Determination of Total Base of Serum*—Add 0.2 ml of serum to the resin in the adsorption tube and follow with two successive 5 ml additions of distilled water Collect the effluent in the test-tube or beaker, and add 1 drop of caprylic alcohol and 1 drop of indicator Then pass a rapid stream of carbon dioxide-free air through the solution for 2 minutes (5 minutes if the carbon dioxide-combining capacity is high) Titrate with

<sup>2</sup> The directions for conversion of the resin from the sodium to hydrogen form are included, since the latter was not available when this study was made If the resin is obtained in the hydrogen form, activation or washing need be carried out only if the test for activity shows the need for it

the standard sodium hydroxide until the color matches that of the control solution. Prepare the latter by adding to a similar test-tube or beaker 0.2 ml of serum, 10 ml of distilled water, and 1 drop of caprylic alcohol. Pass carbon dioxide-free air through the solution rapidly for 5 minutes. Add 1 drop of indicator. It has been the practice in this laboratory to make at least two determinations on each serum.

A standard consisting of 0.2 ml of 0.15 M sodium chloride solution, treated as described above, is included with each set of determinations. A correction is applied for any deviation from the theoretical.

Base combined as bicarbonate is obtained by means of one of the methods described by Van Slyke and Cullen (5) for measuring the carbon dioxide-combining capacity of plasma. Greater accuracy, if required, can be obtained by use of the anaerobic technique for blood collection and actual determination of bicarbonate by methods described by Peters and Van Slyke (6).

*Calculation*—
$$\left( \frac{\text{Normality of base}}{\text{Volume of serum}} \times \text{ml NaOH} \times 1000 \right) + \text{CO}_2\text{-combining capacity of serum (miliequivalents per liter)} - \text{standard salt correction} = \text{miliequivalents total base per liter serum}$$

### Results

*Precision*—On the basis of twenty determinations on one specimen and 126 determinations on samples selected at random, the standard deviation of the mean was found to be  $\pm 1.22$  miliequivalents per liter of serum.

*Accuracy*—To determine the accuracy of the method, simultaneous analyses were made by the ion exchange method and by the electrodialysis procedure of Keys. The results (Table I) showed satisfactory agreement between the two methods in those instances in which the carbon dioxide-combining capacity of serum was higher than 10 miliequivalents per liter. Differences were well within the limit of uncertainty (3 times the standard deviation). There were, however, marked deviations from the electrodialysis method when the carbon dioxide-combining capacity was less than 10 miliequivalents per liter. Agreement between the two methods was not appreciably improved by using brom-thymol blue instead of phenol red as an indicator to give sharper end-points in the pH range of these more acid specimens.<sup>3</sup> An observation that may have some bearing on these discrepancies was the consistent occurrence of the most marked deviations between the two methods when the patients were in terminal states. As an indication of a poor prognosis this finding held remarkably

<sup>3</sup> The discrepancies were too great to be caused by error in the bicarbonate estimation arising from use of calculated rather than determined corrections for carbonic acid and dissolved carbon dioxide.

well Consolazio and Talbott suggested the presence in serum of an unidentified organic or volatile base other than ammonia. They encountered three patients in whom the results of repeated determinations of the total base of serum by electrodialysis were considerably in excess of those determined by analysis of individual ions present. When they asked

TABLE I

*Comparison of Total Base of Serum Determined by Resin Adsorption and Electrodialysis Methods*

The values are given in milliequivalents per liter

Patient No	Bicar bonate	Total base resin method	Total base electrodialysis	Patient No	Bicar bonate	Total base resin method	Total base electrodialysis
1	2 3	145	139	20	13 5	145 9	143
2	4 1	130 8	136 3	21	13 5	156	158
3	4 5	141	144	22	14 4	143 2	139
4	5 0	150	154 5	23	15 5	148 8	152 4
5	5 0	154	153	24	17 4	141	143
6	5 0	152 9	152	25	18 0	146 9	147 1
7	5 0	131	138	26	18 0	163 4	161
8	5 4	147	144	27	18 0	141	142
9	6 3	137 5	141 9	28	18 9	153 3	152 4
10	6 8	144 6	150 5	29	20 3	153	154
11	6 8	155 7	154	30	20 3	146	146
12	6 8	139	142	31	22 5	142 9	140 7
13	7 2	144	154	32	23 0	154	152
14	7 2	169	186	33	23 2	152 4	152 9
15	7 2	129	137	34	23 9	147	146
16	7 0	138	143	35	26 1	138	137
17	7 2	144	141	36	26 6	152 2	150
18	8 1	151	153	37	27 0	151	152 1
19	8 6	174	175	38	27 3	150	153
				39	29 3	148	148
				40	29 6	157 6	158
				41	31 1	149	151
				42	32 0	141	143
				43	32 9	134 3	134 9
Mean		146 2	149 4			148 2 $\pm$ 0 42*	148 2 $\pm$ 0 43*

\*Standard error of the mean

the samples, they obtained agreement between the results with electrodialysis and the sum of the individually determined ions. Our investigation of the samples showing similar deviations was hampered by the small quantities of blood available and the short period of survival of these acidotic patients who were *in extremis*. It was not possible to determine



the individual cations present, nor to carry out measurements on ashed samples. However, the explanation offered by Consolazio and Talbott for their observed differences could well apply. Furthermore, where there was marked lowering of the total base value as determined by means of ion exchange as compared with electrodialysis, the lower value conformed more nearly with expectations based upon determination of the principal anions.

#### SUMMARY

A rapid method for the determination of the total base of serum is described, based on the exchange of hydrogen ions for cations by a synthetic resin and the titration of the eluted anions with standard base. Base bound as bicarbonate is determined separately by gasometric measurement of the carbon dioxide-combining capacity of the serum.

The precision of the new method, with a standard deviation from the mean of  $\pm 1.22$  milliequivalents per liter, is equal to that of existing methods for total base. Results by the new method agree closely with those obtained by the electrodialysis method when the bicarbonate exceeds 10 milliequivalents per liter. Large deviations between the two methods have been observed in some individuals with severe acidosis. These differences disappear if the sample is ashed before electrodialysis.

*Addendum*—Since submitting the paper for publication, it has been possible to examine larger quantities of serum from four patients in diabetic acidosis. Ashing the serum caused no difference in the total base, found by the electrodialysis method, of two of the patients, but in the other two values after ashing were lower by 6 and 9 milliequivalents per liter. In each instance, results given by the resin method agreed with those obtained by electrodialysis of the ashed material.

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## THE RELATIONSHIP OF METHIONINE TO FATTY LIVER PRODUCTION AND GROWTH

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McHenry and Patterson, in a recent review on lipotropic factors (1), have pointed out that there is some disagreement in the literature concerning the lipotropic action of cystine, methionine, and protein Channon, Mills, and Platt (2) have presented additional evidence for the view (3, 4), that there are factors in protein other than cystine and methionine which influence the level of liver fat in rats receiving diets low in choline and high in fat However, in the same communication other experiments are described which confirm the findings of Tucker and Eckstein (5) and Tucker *et al* (6) that the lipotropic effects observed with various low choline diets could be explained on the basis of the methionine and cystine contents Treadwell *et al* (7) found that supplements of methionine as the free amino acid were more effective in preventing the antilipotropic action of cystine than equivalent amounts of methionine in the form of the protein, casein

The study presented below furnishes data relating to three general points in the fatty liver problem about which we wished additional information

1 Treadwell *et al* (7) suggested that an explanation for the superiority of free methionine in comparison to methionine in the form of casein was that the supplementary protein (including methionine) was used in growth Rats receiving diets with high casein levels exhibited a normal weight increase, in marked contrast to the animals receiving the unsupplemented 5 per cent casein diet, which lost weight It seemed unlikely that combined methionine would act qualitatively in a different manner from free methionine If growth is a factor in determining the availability of ingested methionine for lipotropic action, then the importance of the growth factor would be determined by the amounts of other essential amino acids present in the diet Arachin appeared to be the most suitable protein to use in studying this relationship inasmuch as the protein and methionine could be incorporated in the diets separately and at the desired levels Arachin is low in methionine and is lipotropically inactive at dietary levels of from 5 to 20 per cent (8) White and Beach (9) have shown that arachin supplemented with suitable amounts of methionine will support normal growth Thus, in a comparison of 5 and 20 per cent arachin diets, supplemented with methionine to give equivalent amounts in each, the

critical factor would be the other essential amino acids contained in the additional 15 per cent of arachin

2 In general, basal diets low in protein produce high levels of liver fat, and lipotropic effects can be most clearly demonstrated when the liver contains a large amount of lipids. Thus, in the majority of the basal diets (1) which have been used in studying lipotropic factors, the protein level has been from 5 to 10 per cent. However, rats commonly lose or exhibit only a slight increase in weight on these diets. In the present experiment, fatty liver production and growth were studied with 5 or 20 per cent arachin in the basal diets.

3 Best and Ridout (10) have suggested that the action of choline as a lipotropic agent is to promote a normal distribution of fat or to increase the utilization of fat. We have been impressed by the small fraction (about 2 per cent) of the ingested fat which accumulates in the liver during an experimental period. Apparently 98 per cent of the ingested fat is deposited or utilized in a normal manner in choline-deficient rats. There have been only a few observations of the effect of choline on depot fat and on fat balance (1). The fat balance appeared to be of special value in this connection and has been determined on the present experimental animals. The food, feces, liver, and carcass were analyzed for total lipids and the amounts stored and utilized calculated. Glycogen, protein, and water were also determined in the livers and carcasses. These data allowed a characterization of the weight gains and losses.

#### EXPERIMENTAL

White rats, weighing approximately 130 gm, of the strain maintained at the Scripps Metabolic Clinic<sup>1</sup> were used. The care and environment of the animals were the same as described previously (11). Animals of both sexes were included in the group and were distributed uniformly as to sex on the different diets. Only in respect to liver glycogen was a difference observed which could be related to sex. In the livers of the female rats, the glycogen level was uniformly lower than in comparable males. The composition of the experimental diets is given in Table I. The arachin was prepared from peanut meal by the method of Johns and Jones (12). The cystine and methionine contents of the diets were calculated from the data of Brown (13). The experimental period was 21 days. Except for 4 days of the period, all of the animals received daily the same amount of food as was ingested by the rat having the lowest food intake the previous day. One rat on Diet 21 ingested a markedly smaller amount of food on 4 days than the others and for those days the amount for the rest of the animals was adjusted to the second lowest food intake.

<sup>1</sup> We are indebted to Eaton M. MacKay for the gift of these animals.

### Methods

The livers were removed from the unfasted animals under sodium amytal anesthesia, frozen in carbon dioxide snow, and reduced to a powder in a crushing machine. Approximately 1 gm was used for the determination of water and nitrogen as described below. Glycogen and total lipids (fatty acids plus non-saponifiable material) were determined on the remaining portion (14). Water was determined by successively heating the tissue to 80° and cooling to room temperature *in vacuo* over calcium chloride until constant weight was attained. The loss in weight was calculated as water. The nitrogen content of the dried material was determined by the macro-Kjeldahl procedure. The factor 6.25 was used to convert nitrogen to protein. The carcasses, including the viscera, were passed through

TABLE I  
Composition of Diets

All diets were supplemented daily with 1 dried yeast tablet (400 mg) and 2 drops of cod liver oil

	Diet 21	Diet 22	Diet 23	Diet 24	Diet 25
	gm	gm	gm	gm	gm
Arachin	5	5	20	20	20
Cellu flour	2	2	2	2	2
Salt mixture*	5	5	5	5	5
Glucose†	48	48	33	33	33
Lard	40	40	40	40	40
Supplemental methionine		0.473		0.392	0.892
Cystine per 100 gm, calculated	0.067	0.067	0.270	0.270	0.270
Methionine per 100 gm, calculated	0.027	0.500	0.108	0.500	1.000

\* Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, **37**, 572 (1919)

† Generously supplied by the Corn Products Refining Company, New York

a fine meat chopper three times, thoroughly mixed, and two samples removed for the determination of water and nitrogen and of glycogen and total lipids. The methods were the same as for the liver tissue. Preliminary experiments indicated that representative samples were obtained by this procedure. The total lipid content of the feces and of the diets was determined by refluxing for 2 hours with 12.5 per cent alcoholic potassium hydroxide and then proceeding as for liver tissue.

Eight animals were analyzed for protein, total lipids, and glycogen at the start of the experiment and the values were used to calculate the amounts of these materials in the experimental animals initially. The amounts found in the whole animals per 100 gm of body weight were protein 17.9 gm, total lipids 8.1 gm, glycogen 0.12 gm.

Apparent differences were analyzed for significance by the *t* method of Fisher (15) and only those showing a *P* value less than 0.01 were considered significant

TABLE II  
*Composition of Livers and Carcasses of Experimental Animals*

The animals received the diets for 21 days. The data are calculated on the basis of the moist weight and of 100 gm of body weight, except for the second line of data under total lipids which is expressed as gm per 100 gm of moist tissue

	Diet 21	Diet 22	Diet 23	Diet 24	Diet 25
No. of rats	7	7	8	8	8
Change in weight * per cent	-5.2 ±0.7	+8.2 ±2.0	+12.0 ±1.8	+30.1 ±3.8	+28.0 ±2.4
Liver					
	gm	gm	gm	gm	gm
Weight	5.97 ±0.29	4.00 ±0.16	7.41 ±0.39	4.37 ±0.26	3.44 ±0.11
Water	2.79 ±0.10	2.62 ±0.12	3.26 ±0.12	2.53 ±0.11	2.31 ±0.07
Protein	0.79 ±0.02	0.73 ±0.02	0.93 ±0.03	0.74 ±0.03	0.69 ±0.02
Total lipids, per 100 gm body weight	1.88 ±0.18	0.43 ±0.03	3.16 ±0.33	0.84 ±0.10	0.24 ±0.02
“ “ “ 100 “ tissue	31.1 ±1.7	10.7 ±0.5	36.6 ±2.1	18.7 ±1.2	7.0 ±0.3
Carcass					
Weight	93.9 ±0.3	96.3 ±0.4	92.6 ±0.6	95.6 ±0.3	96.5 ±0.1
Water	57.5 ±0.7	58.3 ±0.7	56.7 ±0.7	57.2 ±0.8	58.0 ±0.8
Protein	16.4 ±0.8	16.3 ±0.5	15.9 ±0.6	17.0 ±0.3	16.9 ±0.3
Total lipids, per 100 gm body weight	12.3 ±0.8	14.6 ±0.8	12.7 ±0.6	14.9 ±1.0	14.8 ±0.8
“ “ “ 100 “ carcass	13.2 ±0.8	15.2 ±0.8	13.7 ±0.7	15.7 ±1.0	15.2 ±0.9

\* Including the standard error of the mean calculated as  $\sqrt{\sum d^2/N - 1}/\sqrt{N}$

### Results

The changes in weight and composition of the livers and carcasses of the experimental animals are summarized in Table II. The rats receiving Diet 21 lost 5.2 per cent of their initial body weight during the 21 day period. The animals on Diets 22 and 23 exhibited a slight increase in

weight, while those on Diets 24 and 25 gained 30 per cent of their original body weight

The average weights of the livers of the animals receiving Diets 21 and 23 were significantly greater than in those on Diets 22, 24, and 25. The water content of the livers of the rats on Diet 23 were distinctly higher than in the other dietary groups. In the animals receiving Diets 21, 22,

TABLE III  
*Storage and Utilization of Foodstuffs by Experimental Animals*

	Diet 21		Diet 22		Diet 23		Diet 24		Diet 25	
	gm	per cent	gm	per cent	gm	per cent	gm	per cent	gm	per cent
Food intake	143		151		154		154		154	
	±4		±3		±0		±0		±0	
Lipids in feces	7.2		6.9		5.7		5.9		5.5	
	±0.3		±0.5		±0.2		±0.2		±0.3	
Storage										
Protein	-1.5	-20.5	1.4	18.2	1.2	3.9	6.0	19.6	5.8	18.8
	±0.3	±3.4	±0.2	±2.9	±0.9	±2.9	±0.8	±2.5	±0.6	±2.0
Lipids*	7.3	16.3	14.9	31.9	12.1	24.6	14.9	30.5	13.6	27.6
	±1.0	±2.1	±0.7	±1.8	±1.2	±2.5	±1.3	±2.6	±0.8	±1.7
Carbohydrate	0.04	0.1	0.05	0.1	-0.07	-0.1	0.02	0.1	0.04	0.1
	±0.03	±0.0	±0.02	±0.0	±0.01	±0.0	±0.01	±0.1	±0.03	±0.1
Calories*	59.7	8.2	140.8	18.6	120.0	15.2	157.6	20.0	149.9	19.0
	±11.7	±1.5	±6.3	±1.2	±17.6	±2.2	±14.4	±1.8	±8.0	±1.0
Utilization										
Protein	8.6	120.5	6.2	81.8	29.6	96.1	24.8	80.4	25.0	81.2
	±0.4	±3.4	±0.3	±2.9	±0.9	±2.8	±0.8	±2.5	±0.6	±2.0
Lipids*	36.7	83.7	31.9	68.1	36.9	75.4	33.9	69.5	35.6	72.4
	±1.2	±2.1	±1.5	±1.8	±1.2	±2.4	±1.1	±2.7	±1.0	±1.7
Carbohydrate	69.1	99.1	73.6	99.9	50.9	100.1	50.8	99.9	50.8	99.9
	±2.1	±0.0	±1.3	±0.1	±0.0	±0.0	±0.0	±0.1	±0.0	±0.1
Calories*	658.6	91.8	618.4	81.4	669.5	84.8	630.9	80.0	641.5	81.0
	±27.0	±1.5	±21.3	±1.2	±18.1	±2.2	±13.0	±1.8	±9.7	±1.0

\* Corrected for fat lost in feces

and 24 the water contents of the livers were intermediate between that found in the rats on Diet 23 and the lowest value which was found on Diet 25. The protein contents of the livers of the animals on the various diets bore the same relationship to each other as in the case of the water content. The lipid content of the livers in the five dietary groups fell in the following order, Diet 23, 21, 24, 22, and 25. There were no significant

differences between the various dietary groups as regards the water and protein contents of the carcasses. The lipid content of the carcasses of the animals on Diets 22, 24, and 25 was approximately 14.8 gm, while in the animals receiving Diets 21 and 23 it was approximately 12.5 gm. The *P* value for the difference between these two groups was 0.05, indicating that the difference was probably significant.

The data on the storage and utilization of the ingested foodstuffs are included in Table III. The animals on Diet 21 lost protein from the body tissues during the period. 3.9 per cent of the ingested protein was stored by those receiving Diet 23, while the rats on Diets 22, 24, and 25 stored approximately 19 per cent. There was significantly less storage of fat in the animals on Diet 21 than in any of the other dietary groups. The rats receiving Diet 23 stored 24.6 per cent of the ingested fat, and approximately 30 per cent was stored by those on Diets 22, 24, and 25. The storage and utilization of carbohydrate were the same in all groups. The rats receiving Diet 21 stored 8.2 per cent of the available calories, this is significantly less than in the other four groups.

#### DISCUSSION

The levels of fat in the livers of the rats on the supplemented and unsupplemented 5 per cent arachin diets are in accord with previous observations on the lipotropic activity of this protein (8). Diet 23, which contained 20 per cent unsupplemented arachin, produced a very high level of liver fat. There were 4 times as much methionine and cystine in this diet as in Diet 21. It is possible that increasing the cystine level 4-fold was more effective in promoting fat accumulation in the liver than the same increase in methionine was in preventing it. However, another possibility more in accord with our previous suggestion (7) is that there was present in the 20 per cent arachin diet a supply of the other essential amino acids, so that the animals were able to use the major part of the dietary methionine in growth. This would allow the cystine to exert its full antilipotropic effect. In accord with the above suggestion, the animals on Diet 23 exhibited an increase in weight of 12.0 per cent, while those receiving Diet 21 lost 5.2 per cent of their original body weight.

Another interesting phenomenon in connection with the animals on Diet 23 was the greater weight of the liver in comparison with those of the rats on the other diets. This was not due to the lipid accumulation alone, for there were also significantly larger amounts of water and protein present. Comparison of the levels of liver lipids in the rats on Diets 22 and 24 shows a distinctly higher level in the latter group. Diets 22 and 24 contained 0.5 gm of methionine per 100 gm of diet, with free methionine constituting the major part in each case. However, Diet 24 contained 15

per cent more arachin than Diet 22 and produced a much greater change in weight. We suggest that a larger proportion of the 0.5 gm of methionine in Diet 24 was used for growth than in Diet 22, thus making less available for lipotropic action. There were 4 times as much cystine in Diet 24 as in Diet 22 and the difference in the levels of the liver lipids might be considered as due to the antilipotropic action of the cystine. However, from previous work (7) it may be considered that this amount of cystine (0.27 gm) is unable to exert an effect on the level of liver fat when present in a 5 per cent protein diet containing 0.5 gm of methionine per 100 gm of diet, as in Diet 22. Thus, if the difference in the levels of the liver lipids between the groups on Diets 22 and 24 is due to a cystine effect, it occurred because there was less methionine available for lipotropic action in Diet 24, which again suggests the influence of growth.

The additional 0.5 gm of methionine in Diet 25 over that in Diets 22 and 24 was in excess of that required for growth. It has been shown by Womack and Rose (16) that 0.5 gm of methionine and 0.1 gm of cystine per 100 gm of diet are necessary to promote optimum growth in the white rat. In accord with this, the additional 0.5 gm of methionine in Diet 25 gave no increase in growth rate over that found with Diet 24, which contained 0.5 gm of methionine and 0.27 gm of cystine. The 0.24 gm of liver fat per 100 gm of body weight found in the animals on Diet 25 was significantly lower than the levels found with Diets 22 and 24. The additional methionine in Diet 25 produced a marked lowering of the liver lipids. The low level of liver fat obtained with Diet 25 is of some interest in view of the findings of Best and Ridout (17) and of Channon, Manifold, and Platt (18) that on various low protein diets the maximum lipotropic effect is obtained when supplementary methionine comprises 0.2 to 0.5 per cent of the diet. Additional quantities of methionine did not cause a further decrease in spite of the fact that large amounts of fat were still present in the liver. The above workers have also observed that progressive increase in the casein content of the diet decreases the liver fat until a level only slightly above normal is obtained. From the present experiment it may be concluded that free methionine, if added in sufficient quantity over that required for growth on a 20 per cent protein diet, will lower the liver lipids to the same extent as casein. The explanation for the effectiveness of methionine (more than 0.5 per cent of diet) when added to a 20 per cent protein diet and the lessened action of comparable quantities in low protein diets will require further study. However, we suggest that it resides in the different nutritional status of animals on high and low protein diets.

The water and protein content of the carcasses of the rats was the same regardless of the diet. The data suggest an increased lipid content of



the carcasses in the animals receiving supplementary methionine. Thus, while the nature of the diet determined the rate of growth, the composition of the newly formed tissue, except for the fat content, was not influenced by the diet. The weight changes may be related to the nature of the diets in the following manner. Diet 21 was deficient in both methionine and protein and a loss in weight of the animals occurred. Diet 22 was deficient in protein. The 5 per cent of arachin, which it contained, could support only minimum growth. Diet 23 contained a sufficient quantity of protein but was deficient in methionine and thus produced only minimum growth. Diets 24 and 25, which contained amounts of methionine and protein sufficient to support normal growth, gave what is probably maximum growth on the restricted food intake of this study. The data may be interpreted as suggesting that the amount of methionine that was utilized in growth was determined by the amounts of the other essential amino acids present in the diet and the extent to which the available methionine was utilized in growth determined the amount available for lipotropic action.

The data summarized in Table III permit some deductions concerning the processes involved in the phenomena of growth and lipotropic action. The changes in weight of the experimental animals were apparently related to the storage and utilization of protein. The initial body protein decreased 20.5 per cent in the rats on Diet 21 which lost weight during the experimental period. Groups on Diets 22 and 23 gained approximately 10 per cent of their initial weight, and stored the same absolute amount of protein. As discussed above, the storage on Diet 22 was limited by the amount of protein and on Diet 23 by the amount of methionine. The storage of protein in the rats receiving Diets 24 and 25, which gained 30 per cent of their initial weight, was the same both in absolute amounts and in percentage of that ingested. The additional 0.5 gm. of methionine in Diet 25 did not increase the storage over that found in Diet 24. Evidently, a storage of approximately 20 per cent of the ingested protein represents the maximum influence of methionine in this regard. This is also suggested by the findings in the animals on Diet 22, which stored 18.2 per cent of the ingested protein, even though there was no deficiency of methionine and the protein intake was at a low level. Also the storage of lipids appeared to be related to the methionine and protein intake, for in the animals on the five diets, the per cent of the ingested lipids stored bore the same relationship to each other as was found among the values for the per cent of protein stored. The carbohydrate storage and utilization were the same in all groups.

The data suggest that the addition of methionine to a methionine deficient diet promotes the storage (deposition) of protein and fat in the

tissues of the growing rat When an adequate amount of methionine is present in the diet, approximately 20 per cent of the ingested protein is deposited and 80 per cent is utilized in processes which involve the disappearance of the contained nitrogen from the body Moreover, while the quantity of dietary methionine appeared to determine the per cent of the ingested protein which was stored, the growth which occurred during the experimental period was limited by the protein intake

If the lipotropic action of methionine is due to the transfer of methyl groups to aminoethanol in the synthesis of choline (19), then the present data do not support the suggestion of Best and Ridout (10) that choline may promote the utilization of fat in the liver In the present study, the data may be interpreted as indicating that methionine decreased the utilization of fat and promoted its deposition in tissues other than the liver The data are not incompatible with the possibility that in the over-all storage and utilization of fat the effect is primarily one in relation to growth and indirectly on the metabolism of fat The data also emphasize the minor importance of the level of the liver lipids in the total fat metabolism of the body The livers containing the largest amounts of lipids occurred in the rats on Diet 23 which were intermediate as regards the proportion of the ingested fat deposited The group on Diet 24, in which fatty livers developed, stored the same percentage of the ingested fat as did the rats on Diet 22 with a low level of liver fat Thus, it is possible to design diets upon which the rats ingesting them will develop fatty livers and still utilize the ingested fat in an essentially normal manner

#### SUMMARY

1 Under conditions obtaining in this study, the amounts of the other essential amino acids present in the diets determined the amount of dietary methionine that was used in growth

2 The amount of dietary methionine available for lipotropic action appeared to be limited by the amount used in growth

3 The rats receiving the diet which contained 20 gm of arachin and 0.5 gm of methionine per 100 gm of diet grew normally and developed fatty livers

4 The rats receiving the diet which contained 20 gm of arachin and 1 gm of methionine per 100 gm of diet grew normally and the level of the liver lipids was only slightly above normal

5 The addition of methionine to a methionine-deficient diet promoted the storage of protein and fat in the tissues

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# GROWTH IN CHICKS FED AMINO ACIDS

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Studies on the amino acid requirements of the chick have been delayed by the complex vitamin requirements of this species. Biotin, in particular, is associated with protein residues, and concentrates of this vitamin were needed before purified, low protein diets could be devised. Such concentrates, and recently crystalline biotin, have been made available. However, by the use of proteins which are low in certain amino acids, such as casein which is low in glycine and arginine and protein hydrolysates from which certain amino acids have been removed, it has been possible to demonstrate a requirement for certain amino acids. Unlike the rat, the chick requires glycine for normal growth (1-3), and arginine is necessary even for maintenance (4). Methionine is required (5) and as in the rat may be replaced with choline and homocystine. Methionine will not, however, completely replace choline (6, 7). Cystine additions give responses on casein diets (8), but studies on cystine-free diets have not yet been reported. Presumably, methionine will replace cystine. Tryptophan is essential (9), and growth responses have been obtained with histidine (4) and lysine (10) additions on diets low in these amino acids. The literature on this problem has been extensively reviewed by Jukes and Almquist (11). No studies have as yet appeared in which crystalline amino acids have been used as the sole source of amino acids for the chick nor is there any evidence indicating the necessity or dispensability of the other amino acids which are essential for the rat, namely leucine, isoleucine, phenylalanine, threonine, and valine. Data presented in this paper show that growth may be obtained in chicks fed crystalline amino acids as practically the sole source of protein and suggest that each of the above five amino acids is also essential in the diet of chicks.

## EXPERIMENTAL

The low protein basal diet<sup>1</sup> used in these studies had the following percentage composition: corn oil 5, salt mixture 5 (12),  $\text{CaHPO}_4$  10, calcium gluconate 20, cod liver oil 20, Cellu flour 20, Wilson's Liver

<sup>1</sup> The liver extract was supplied by Dr. David Klein of The Wilson Laboratories, Chicago. The crystalline vitamins were furnished by Merck and Company, Inc., Rahway, New Jersey.

those which received no protein supplements, although no specific symptoms other than extreme weakness and a tendency toward cannibalism were noted

Of the two chicks receiving no glycine, one maintained its weight while the other gained slowly

#### DISCUSSION

Although the basal diet is not protein-free, it is unlikely that it contains sufficient nitrogen to supply the requirement of any one amino acid. Thus the amino acid mixture probably contains all of the amino acids which are essential for growth in chicks. Glycine appears to be non-essential in the strict sense, since weight maintenance and some growth are possible without it. It is probably synthesized to a limited extent.

Kinsey and Grant (17) have shown, contrary to the results of Albanese and Irby (18), that growth may be obtained in rats fed only the essential amino acids as a source of protein. These investigators found that at a suboptimum level of nitrogen the growth obtained upon a mixture of the essential amino acids was similar to that obtained with casein. It would be of interest to know whether this is true when only the essential amino acids are fed, but at an optimum nitrogen level. In other words, can the body synthesis of all of the non-essential amino acids keep pace with the demands for optimum growth? In his studies on the rat, Rose (15) has defined an essential amino acid as one which cannot be synthesized by the animal organism out of the materials *ordinarily available* at a speed commensurate with the demands for *normal* growth. He has pointed out that there may well be quantitative differences in the synthetic powers of the organism, ranging from those amino acids which cannot be synthesized at all (perhaps not rapidly enough for maintenance of weight or life) through amino acids such as arginine, which are made slowly, to those which may be synthesized at a rate commensurate with normal growth. However, normal growth has probably never been obtained on diets in which the sole source of nitrogen was supplied by amino acids. It is possible and even probable that other amino acids may fall in the same category as does arginine although synthesized at a somewhat more rapid rate. Thus the inclusion of arginine in the list of essential amino acids for the rat to the exclusion of others may be artificial in that experimental conditions have not as yet permitted the demonstration of the rate of synthesis of the other amino acids.

The failure to obtain good growth in chicks on the amino acid mixture used in these experiments can scarcely be attributed to a lack of nitrogen for the synthesis of the non-essential amino acids nor to suboptimum amounts of the amino acids that were fed. Toxicity of the amino acids

at this level must be considered but appears less likely than the possibility that the chick has limited synthetic powers for one or more of the amino acids which were not supplied. The data of Kinsey and Grant suggest that this may be true for the rat as well. Since a 10 per cent casein diet is deficient in methionine and perhaps in other essential amino acids, one might expect to get superior growth on the same level of nitrogen supplied by only the essential amino acids.

It would appear advantageous to define essential amino acids as those essential for life. Certain amino acids would then be classified as stimulatory, or necessary for normal growth. These must for the present be determined by experimentation, but certainly arginine would be so classified for the rat. This may seem unduly complicated but has proved to be the only practical method of classification in similar situations commonly met in bacterial nutrition. The border line between an essential amino acid and a stimulatory one may be so close as to be controversial. However, this type of classification would be advantageous in that at present undue emphasis is thrown upon the essential amino acids. It has been stated (19) that "the biologic value of each of the proteins in food is also dependent on the relative composition of these essential amino acids." However, it is well known that cystine is not essential, yet in the absence of sufficient methionine is fully as important as the essential amino acids. Thus cystine may be an important amino acid in practical nutrition, and a similar situation with regard to other non-essential amino acids seems possible.

It should be noted that failure to obtain growth or to maintain nitrogen balance when a single amino acid is omitted from a mixture consisting of a limited number of amino acids, as was done in this and other experiments (20), does not necessarily prove their indispensability. The possibility still remains that they may be replaceable or interchangeable with other amino acids. Burroughs *et al* (21), for example, state that norleucine may function in the adult rat in promoting synthesis of lysine or of leucine or of both, though this is clearly not the case in young growing rats (22, 23). Thus extensive studies with complete mixtures of amino acids, as exemplified by the studies of Rose on growing rats, will be required before the essential amino acids of each new species can be stated with assurance.

#### SUMMARY

A diet containing a mixture of amino acids, consisting of leucine, isoleucine, threonine, phenylalanine, valine, methionine, tryptophane, cystine, arginine, lysine, histidine, and glycine as the only substantial source of nitrogen, allows growth in the chick, although much less than a comparable level of adequate protein. The omission of leucine, isoleucine, threonine,

phenylalanine, or valine from this mixture resulted in failure of growth and loss in weight. When glycine is omitted, maintenance of weight or slight growth is still possible.

These data suggest that leucine, isoleucine, threonine, phenylalanine, and valine are essential amino acids for the chick. Glycine is required for normal growth.

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# MODIFICATION OF THE KING-ARMSTRONG METHOD FOR THE DETERMINATION OF PHOSPHATASE\*

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The King-Armstrong method for the determination of phosphatase values of serum and plasma (1) has been modified in this laboratory so that 1 cc. of plasma or serum suffices for the determination of both acid and alkaline phosphatases. In addition, a more convenient standard has been used to facilitate the colorimetric determination. In the original method the phenol released by the action of phosphatase upon phenyl phosphate is measured colorimetrically. It was necessary, therefore, to prepare a standard solution of phenol. The phenol content of the solution was determined by iodometric titration. We have found it convenient to use, instead, a solution of tyrosine as the standard. Tyrosine may be obtained in pure anhydrous form and is, therefore, a more convenient primary standard.

The phosphatase values of plasma determined with phosphotyrosine as a substrate have not been found to vary significantly from those for which phenyl phosphate was used. While phosphotyrosine is not commercially available, it may be readily prepared by the method of Plummer (2). The use of the phosphotyrosine as a substrate does not, in any way, modify the technique of determination. Since it is our belief that physiological substrates should be used whenever possible in the determination of enzyme concentrations, we believe phosphotyrosine to be a preferable substrate.

## EXPERIMENTAL

*Reagents*—The buffer solutions and the disodium phenyl phosphate solution are identical with those used in the King-Armstrong (1) and Gutman-Gutman (3) techniques. In the proposed method 1 part of the substrate solution is mixed with 9 parts of the buffer solution.

*Disodium phenyl phosphate* 1.09 gm. of disodium phenyl phosphate

\* The Bureau of Medicine and Surgery does not necessarily endorse views or opinions expressed in this paper.

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§ L. Q. Forman, Ph. M. 2/c U. S. N. R., gave valuable technical assistance.



are dissolved in 500 cc of water This solution keeps well if stored in a refrigerator

*Phosphotyrosine* 1.30 gm dissolved in 500 cc of water This solution should be stored in a refrigerator

*Acid buffer solution* 42.0 gm of citric acid are dissolved in water 376 cc of 1.0 N sodium hydroxide are added and the mixture is diluted to 1 liter The pH is adjusted to 4.9 This reagent is stored in a refrigerator

*Alkaline buffer solution* 10.3 gm of sodium diethyl barbiturate are dissolved in water and diluted to 500 cc

*Phenol reagent of Folin and Ciocalteu* (4) Dilute 1 part of the phenol reagent with 3 parts of approximately 15 per cent hydrochloric acid before use The hydrochloric acid is prepared by mixing 5 cc of concentrated hydrochloric acid and 95 cc of water

*Sodium carbonate* A 20 per cent solution

*Stock standard* Exactly 1.926 gm of tyrosine are placed in a 1 liter volumetric flask and dissolved by the addition of 8.5 cc of concentrated hydrochloric acid in 200 cc of water The solution is diluted to 1 liter with water

*Dilute standard* 1 cc of the stock standard is diluted to 250 cc with water Each cc is equivalent to 0.004 mg of phenol

*Alkaline buffer-substrate solution* 1 part of the substrate solution is mixed with 9 parts of the alkaline buffer immediately before use

*Acid buffer-substrate solution* 1 part of the substrate solution is mixed with 9 parts of the acid buffer immediately before use

### Procedure

*Acid Phosphatase*—Four small test-tubes, each containing 5 cc of the buffer-substrate solution, are kept in a water bath at 37° for about 5 minutes 1 cc of the test plasma is mixed with 9 cc of physiological saline and 1 cc of the diluted plasma is added to each of two tubes The contents are mixed and the incubation is allowed to continue for 3 hours The tubes are removed from the water bath and 2 cc of the diluted phenol reagent are added to all four tubes To the two tubes which serve as a control is added 1 cc of the diluted test plasma The contents of all the tubes are mixed and filtered

5 cc from each filtrate are transferred to a colorimeter tube and 2.0 cc of 20 per cent sodium carbonate are added The contents are mixed and the color is allowed to develop at 37° for 1 hour The color is then determined in a photoelectric colorimeter in which a light source of 660 m $\mu$  is used

*Alkaline Phosphatase*—The incubation period is exactly 30 minutes

with the alkaline buffer-substrate mixture, but, otherwise, the procedure is identical with that for acid phosphatase described above

*Calculation of Results*—A standard curve is prepared in which samples of the dilute standard containing from 0.004 to 0.024 mg of phenol equivalent are diluted to 6 cc with water. 2 cc of the diluted phenol reagent are added, the solution is mixed, and 3.2 cc of the 20 per cent sodium carbonate solution are added. The contents of the tubes are mixed and, after 1 hour incubation at 37°, the color is determined in a photoelectric colorimeter.

The results are expressed as units of activity in 100 cc of plasma or serum. A unit of acid phosphatase is that amount of activity which will liberate 1 mg of phenol in 1 hour. A unit of alkaline phosphatase is that amount

TABLE I

*Comparison of Values Obtained with Disodium Phenyl Phosphate and Phosphotyrosine As Substrates*

The values are given in units which represent mg of phenol or phenol equivalent released by 100 cc of serum in 1 hour for the acid phosphatase and in 30 minutes for the alkaline phosphatase.

Case No	Acid phosphatase		Alkaline phosphatase	
	Disodium phenyl phosphate	Phosphotyrosine	Disodium phenyl phosphate	Phosphotyrosine
1	0.4	0.7	3.2	2.2
2	0.5	0.7	2.0	1.6
3	1.1	1.8	4.6	3.9
4	1.3	1.6	7.0	6.2
5	1.7	1.9	7.0	6.4
6	2.0	2.3	10.2	9.6
7	2.3	2.6	15.8	14.9
8	2.4	2.6	11.4	10.8

of activity which will liberate 1 mg of phenol in 30 minutes. The values for the control tube in units, or in mg of phenol, are subtracted from the total value. 0.004 mg of phenol is equivalent to 4 units of alkaline phosphatase or to 1.33 units of acid phosphatase. It is convenient to express the standard curve in units of phosphatase activity.

#### DISCUSSION

The results given in Table I are typical of those obtained on a variety of sera in which the two substrates were compared. The acid phosphatase values with phosphotyrosine as the substrate are consistently but hardly significantly higher than those with disodium phenyl phosphate. The alkaline phosphatase values are, on the other hand, slightly lower when phosphotyrosine is used as the substrate.

Values for normal adults have been found to fall within a range of 1.5 to 8 units of alkaline phosphatase. The alkaline phosphatase values of children are somewhat higher and are within a range of 4 to 14 units. Normal acid phosphatase values for the adult have been found to be 2 or less units. When alkaline phosphatase values greater than 20 are encountered, it has been found necessary to repeat the determination and use a greater dilution of the serum. A dilution of 1 cc of plasma with saline to a total volume of 100 cc has been found satisfactory in all cases of high phosphatase values.

#### SUMMARY

A modification of the King-Armstrong method of phosphatase determination has been described in which both acid and alkaline phosphatase values of serum and plasma may be determined with 1 cc of plasma or serum. Tyrosine has been used as a convenient standard for the colorimetric estimation of substrate attacked. Phosphotyrosine has been used as an alternate substrate.

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# FURTHER STUDIES OF PYRUVATE METABOLISM BY LIVERS FROM VITAMIN B-DEFICIENT RATS\*

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Previous work (1) has shown that the rate of oxygen consumption in the presence of pyruvate by homogenates of liver from pantothenic acid- and biotin-deficient rats was decreased below that of normal rats. More detailed investigations have confirmed the previous results and have more clearly defined the conditions under which the effect is obtained. It has also been shown that the pyruvate metabolism of livers of riboflavin- and thiamine-deficient rats is not markedly decreased under these conditions. However, subsequent studies on rats maintained on other regimens indicate that there may be some question about the specificity of the effect of the pantothenic acid and biotin deficiencies, and that the mechanism of the effect is more likely an indirect one.

## EXPERIMENTAL

*Treatment of Animals*—Weanling, male, albino rats of the Sprague-Dawley strain were placed on the purified rations and supplements previously described (1). In addition to the biotin and pantothenic acid deficiencies, thiamine and riboflavin deficiencies were produced by omitting the respective vitamin from the control supplement.

The animals were maintained on the diets for 8 to 12 weeks, except for the thiamine-deficient rats which could be kept for only 4 to 5 weeks before death ensued. After 3 weeks on experiment the control rats were restricted to a minimum of 4 to 5 gm. of ration per day to limit their growth to that of the deficient groups. The deficient animals were fed *ad libitum*.

In the experiments of Series II, respiration studies were made on the livers of rats fed rations similar to those above but containing (a) 0.75 per cent sulfasuxidine (succinylsulfathiazole), (b) 0.75 per cent sulfathiazole, (c) 0.50 per cent sulfadiazine, (d) 0.50 per cent thiourea, (e) 1.00 per cent *p*-aminobenzoic acid. All rations were fed *ad libitum* and the vitamin

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supplement contained, in addition to the six crystalline B vitamins, 1  $\gamma$  of biotin per day (S M A concentrate No 1000) The rats also received the fat-soluble vitamins, A, D, E, and K The complete details of the rations and the supplements are given in a report on a study on the sulfonamides<sup>1</sup> The respiration measurements were made when the rats had been on experiment for 6 to 10 weeks

*Respiration Studies*—All measurements of oxygen consumption were performed in the Warburg respirometer at 37.5° in a total volume of 30 ml, and with 0.2 ml of 10 per cent KOH and a strip of filter paper in the center well to absorb the carbon dioxide evolved

TABLE I

*Pyruvate Metabolism of Liver Homogenates of Pantothenic Acid-Deficient and Control Rats in Presence and Absence of Magnesium Ions*

Group	No of rats	Magnesium ions present 0.0013 M		No magnesium ions	
		60 min oxygen uptake	Pyruvate removed in 60 min	60 min oxygen uptake	Pyruvate removed in 60 min
		microliters	microliters	microliters	microliters
Pantothenic acid-deficient	7	293 (202-346)	342 (238-440)	181 (167-191)	225 (188-256)
Control (restricted food intake)	5	333 (295-369)	474 (404-540)	334 (263-343)	477 (353-540)

The "60 minute oxygen uptake" is the oxygen consumption per 100 mg of wet tissue extrapolated back to zero time in order to compare it with the amount of pyruvate removed

"Pyruvate removed" is expressed as microliters of CO<sub>2</sub> according to the reaction, CH<sub>3</sub>COCOOH  $\rightarrow$  CO<sub>2</sub> + CH<sub>3</sub>CHO, for the amount of tissue and the time corresponding to the "60 minute oxygen uptake"

The original buffer and quantity of substrate were modified so that each flask had one of the following sets of conditions (1) *no magnesium*, 0.067 M sodium phosphate buffer, 0.5 ml, 0.49 M sodium chloride, 0.5 ml, (2) *plus magnesium*, same as (1) plus 0.2 ml of 0.02 M magnesium chloride or sulfate, (3) *borate*, 0.4 M sodium borate buffer, 0.3 ml In addition, each flask contained 0.4 ml of 0.2 M sodium pyruvate and 1.0 ml of a 10 per cent homogenate (2) of liver in 0.0167 M sodium phosphate buffer and 0.12 M sodium chloride The final pH in the flasks was 7.4 to 7.5 The results (except in Table I) are expressed as Q<sub>0</sub>, (microliters of oxygen consumed per mg of dry weight of tissue per hour) for the 10 minute intervals following the 10 minute equilibration period

*Pyruvate Utilization*—Pyruvate removal was measured by adding a definite quantity and then determining the amount remaining after a

given period of respiration. Several methods were available for the determination of pyruvate (3-5), but all of them had certain limitations. Westerkamp's method (5), in which a yeast extract is employed, suggested the possibility of using a washed, dried yeast. Consequently an apozymase preparation (6) was tested under various conditions and the following method adopted. The method of preparing the sample for analysis is a modification of the procedure of Evans (7).

At the conclusion of the respiration measurements, the flasks were placed in ice water and 0.20 ml of 2.0 N acetic acid was added to stop the reaction and precipitate the proteins. This suspension was transferred quantitatively to a graduated centrifuge tube, diluted to about 6.5 ml, and centrifuged for 10 to 15 minutes. 0.20 ml of 1.0 N NaOH was then added, and the volume adjusted to 6.8 ml. The final pH was 5. An aliquot of 1.7 ml was placed in the respirometer flask, and a Keilin cup (8) containing 60 mg of apozymase and 15  $\gamma$  of cocarboxylase in 0.3 ml of 0.02 M acetate buffer, pH 5, was suspended from the center well. The respirometer was equilibrated at 30° for 15 minutes, the cup tipped in, and the total carbon dioxide evolution determined. The yeast carboxylase catalyzes the following reaction:  $\text{CH}_3\text{COCOOH} \rightarrow \text{CO}_2 + \text{CH}_3\text{CHO}$ . Pyruvate utilization is expressed as carbon dioxide equivalent to that evolved according to this reaction. Recovery was 98 to 103 per cent after correction for an endogenous evolution of gas by the apozymase in the absence of added pyruvic acid.

### Results

*Series I*—The 10 minute  $Q_{O_2}$  values for the livers of the pantothenic acid- and biotin-deficient and of the restricted control animals are plotted in Fig. 1. The 10 minute and the 40 minute averages with the ranges for these groups and for the riboflavin- and the thiamine-deficient groups are given in Table II. Because the first publication (1) listed only the net  $Q_{O_2}$  (pyruvate) values, these original figures are given in Table III along with the total  $Q_{O_2}$  (pyruvate) values for purposes of comparison. These total values (obtained in a buffer comparable to the present, *plus magnesium* buffer) compare favorably with the figures for the *plus magnesium* buffer in Table II and Fig. 1. The total oxygen uptake for 60 minutes and the amount of pyruvate utilized, expressed as microliters of carbon dioxide, in the 60 minute interval, for the pantothenic acid-deficient and the control rats, are summarized in Table I. These values show that the pyruvate removal correlated better with the total than with the net oxygen uptake. This correlation was confirmed by experiments performed in the *borate* buffer, which suppressed the endogenous respiration almost completely, nevertheless, the oxygen consumption in the presence of pyruvate and the

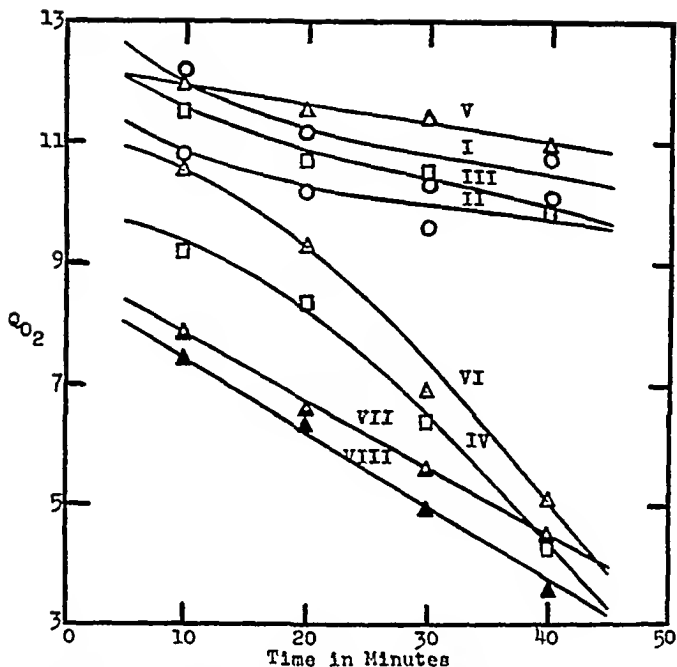


FIG 1 Average  $Q_{O_2}$  values of homogenates of livers of rats exhibiting one of various vitamin B deficiencies in the presence and absence of magnesium. Curve I, basal control, no magnesium, Curve II, same, plus magnesium, Curve III, pantothenic acid deficiency, no magnesium, Curve IV, same, plus magnesium, Curve V, biotin deficiency (Group 2), no magnesium, Curve VI, same, plus magnesium, Curve VII, biotin deficiency (Group 1), no magnesium, Curve VIII, same, plus magnesium

TABLE II

*Pyruvate Metabolism of Liver Homogenates from Vitamin-Deficient Rats in Presence and Absence of Magnesium Ions*

Group	No of rats	No magnesium		Magnesium present, 0.0013 M	
		0-10 min	30-40 min	0-10 min	30-40 min
Pantothenic acid-deficient	7	$Q_{O_2}$ 11.47 (8.7-14.3)	$Q_{O_2}$ 9.89 (6.2-12.1)	$Q_{O_2}$ 9.27 (7.9-10.5)	$Q_{O_2}$ 4.24 (3.8-6.2)
Biotin-deficient, Group 1	6	7.90 (6.7-9.6)	4.43 (3.0-6.1)	7.44 (6.2-9.5)	3.63 (2.9-4.6)
" " 2	2	11.90 (9.6-14.2)	10.90 (10.5-11.2)	10.70 (9.8-11.6)	5.00 (5.0-5.0)
Restricted controls	5	12.11 (10.6-13.8)	10.68 (9.9-11.2)	10.80 (9.8-11.8)	10.08 (8.3-10.4)
Riboflavin-deficient	3	9.60 (9.2-9.8)	9.87 (8.7-10.9)	8.43 (8.4-8.5)	8.23 (7.5-9.0)
Thiamine-deficient	2	9.70 (9.2-10.2)	8.30 (7.1-9.5)	7.85 (7.2-8.5)	6.95 (6.9-7.0)

amount of pyruvate removed were the same as those obtained with the phosphate buffer. Therefore, in the evaluation of the results, the endogenous respiration was not considered because it apparently had no significance. This obviated fasting the rats before decapitation, which was previously done in an effort to decrease the endogenous respiration.

The rates of oxygen uptake of the tissues from the various groups of animals in the sodium chloride-sodium phosphate buffer in the presence of and absence of magnesium ions are given in Fig 1 and in Table II. An examination of these figures and the values for the amount of pyruvate utilized (Table I) shows that the changes observed are more pronounced in the presence of magnesium ions. In an effort to determine whether this effect was specific for the deficiencies originally investigated or whether the lack of the effect in the controls was the result of their partial fasting, the effect of magnesium ions was tested on the livers of animals deficient

TABLE III

*Q<sub>O<sub>2</sub></sub>* Values for Liver Homogenates from Pantothenic Acid- and Biotin-Deficient Rats

Group (12 rats in each)	0-20 min		20-40 min	
	Total	Net	Total	Net
Pantothenic acid-deficient	7.59 (5.0-10.0)	2.20 (0.2-4.6)	4.14 (2.4-5.7)	0.93 (-0.3 to 1.6)
Biotin-deficient	7.73 (5.7-9.3)	2.23 (0.4-3.9)	5.37 (3.9-7.0)	1.40 (0.3-3.1)
Controls	12.23 (8.7-15.8)	6.48 (2.8-10.4)	12.20 (7.3-16.2)	8.50 (4.9-13.1)

Total  $Q_{O_2}$  is in the presence of pyruvate. Net  $Q_{O_2}$  is the total  $Q_{O_2}$  minus the endogenous  $Q_{O_2}$ .

in riboflavin and thiamine, respectively. It is evident from the  $Q_{O_2}$  values that the degree of magnesium inhibition was very pronounced in the pantothenic acid and biotin deficiencies but was relatively small in the controls and in the other deficiency groups. Furthermore, in the biotin-deficient animals, there was a rapid decrease in the metabolic rate in most of the livers even in the absence of magnesium. These animals were divided into two groups in Fig 1 and Table II: (a) those that showed a decrease in the absence of magnesium (Group 1, Curves VII and VIII), and (b) those that showed the effect only in its presence (Group 2, Curves V and VI).

*Series II*—The decrease in respiration rate in the presence of magnesium ions was somewhat variable in several of these groups. The controls fed *ad libitum* exhibited a trend similar to that of the restricted controls in Series I, but the rate was not so uniformly high. The livers of rats fed sulfadiazine showed the magnesium inhibition observed in the panto-



themic acid- and biotin-deficient animals. On the other hand, the livers of those fed *p*-aminobenzoic acid or thiourea showed a range from the slight inhibition characterizing the controls to an inhibition which was quite marked, although not usually as great as that in the pantothenic acid- and biotin-deficient animals. The rats given sulfasuxidine or sulfathiazole have not been included in this report because of the extreme inhibition of several enzyme systems caused by a high calcium content of their livers<sup>1</sup>

#### DISCUSSION

The use of the total rather than the net  $Q_0$  value in the presence of a substrate for the evaluation of measurements of enzymatic activity is more likely to be quantitatively correct. This fact is evident in this investigation because of the correlation of the total  $Q_0$  (pyruvate) with the amount of substrate utilized in several different media. Without giving specific data, Potter has emphasized this point in his recent review ((9) p 204)

The decrease in the rate of pyruvate metabolism in the presence of magnesium ions, reported here, is at variance with the generally accepted view that magnesium is an activator of the enzymes metabolizing pyruvate. It may be mentioned at this point that manganous ions were found not to have this inhibitory effect, although they are usually interchangeable with magnesium ions in pyruvate metabolism. The inhibition by magnesium may conceivably be an indirect effect which outweighs its direct effect as an activator under the conditions used in these experiments. For example, this might be so if the adenosine triphosphate-adenosinetriphosphatase system were a limiting factor. Potter has indicated that pyruvate metabolism requires adenosine triphosphate ((9) p 238), other experiments have shown that adenosinetriphosphatase is inhibited by magnesium ions (10). If this system in the rat liver requires adenosine triphosphate, but no additional amounts are added, it could be a limiting factor in the deficient animals which is aggravated by the inhibition of the system by magnesium, while in the controls the supply of adenosine triphosphate (originally present and resynthesized) and the activity of the adenosinetriphosphatase may be sufficient to maintain the system even in the presence of magnesium.

The variation within groups, observed in Series II, may conceivably be due to (a) the nutritional status of the animals, or (b) the incompleteness of the enzyme system or to both. Animals fed purified rations with only known constituents are not in a state of optimum nutrition. The sulfadiazine and the biotin and pantothenic acid deficiencies may decrease the

<sup>1</sup> Pilgrim, F. J., and Elvehjem, C. A., unpublished work.

production or utilization of some unknown factor, nutritional or otherwise. This may be only partially true in the rapidly growing groups. On the other hand it may be adequate for the restricted controls and for the riboflavin- and thiamine-deficient rats.

Secondly, on the basis of more recent evidence, the enzyme system is not complete. Potter has shown that pyruvate metabolism *in vitro* requires, among other factors, coenzyme I, adenosine triphosphate, and glutathione ((9) p 238). Although this system functions in rabbit and guinea pig kidney, it does not in rat kidney. Because this system is not completely defined in rat liver either, there may be more than one variable affecting the results.

The marked difference between the respiration rates in the presence of magnesium ions in the livers of the pantothenic acid- and the biotin-deficient rats on the one hand and the restricted controls and riboflavin- and thiamine-deficient rats on the other hand still suggests the possibility of some function, more likely indirect, of pantothenic acid and of biotin in pyruvate metabolism in rat liver. The final solution of this problem awaits further knowledge of nutritional requirements, but, more particularly, a more completely characterized enzyme system.

#### SUMMARY

1 Pyruvate oxidation by homogenates of liver from rats in various nutritional states was measured in the presence and the absence of magnesium ions in a phosphate buffer.

2 The presence of magnesium caused a decreased  $Q_{O_2}$  (pyruvate) in the livers from rats deficient in pantothenic acid or biotin, and in those fed sulfadiazine.

3 In the case of riboflavin- and thiamine-deficient rats and normal rats fed restricted quantities of purified ration, the magnesium caused no significant inhibition.

4 The results were variable when the rats were fed *p* aminobenzoic acid, thiourea, or the basal ration *ad libitum*.

5 The results summarized in (2) and in (3) suggest that biotin and pantothenic acid have some function, direct or indirect, in pyruvate metabolism.

6 Summary (4) indicates that the results are complicated by complex nutritional requirements and a complex enzyme system, neither of which is completely known at present.

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# CHOLINE AND THE PREVENTION OF HEMORRHAGIC KIDNEYS IN THE RAT

## III AMOUNTS OF WATER, NITROGEN, TOTAL LIPID, AND CHOLINE IN LIVERS AND KIDNEYS

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In the course of an investigation of the production of hemorrhagic kidneys by the maintenance of rats upon a choline-free diet it has been found that (a) the damaged kidneys have a decreased concentration of phospholipids coincidental with a similar decrease in the liver (1), (b) the rate of phospholipid turnover is normally high at that period in the rat's life in which choline deficiency most easily produces kidney damage (2), and (c) the renal lesions can be prevented by triethylcholine (1, 3), a compound incapable of supplying methyl groups (4) but active as a lipotropic agent (5). These results supported a hypothesis that the dietary lack of choline or of choline precursors, interfering with the formation of phospholipid during a period of rapid growth, caused degeneration in kidney structure. The present report deals with additional data in support of this explanation.

### Methods

The age, weight, sex, diet, and vitamin supplements of the rats were the same as previously reported (1). The kidneys from animals maintained on the basal diet with or without choline were analyzed for water, nitrogen, and fat on the initial, 7th, or 10th experimental day. The livers from the same animals were analyzed for water and fat.

The amount of moisture was determined by drying the finely minced organ to constant weight on a Petri dish in an oven at 90°. Nitrogen was determined on the dried sample by the micro-Kjeldahl method. The amount of total lipid was determined by ether extraction of the dried sample in a Soxhlet apparatus. This total lipid will subsequently be designated as "fat." Phospholipids were determined by the method of Bloor (6). Choline estimations were made by the method of Engel (7). All analyses were made on tissues pooled from each group of animals; previous work had shown that results secured by the use of individual organs showed little overlapping.

### Results

In all of the animals deprived of choline marked degeneration of the kidneys was evident. The abnormalities were the same as those described by Griffith and Wade (8).

Table I shows the composition of the kidneys

The changes in the composition of the kidney produced by choline deficiency are of considerable interest. During the 10 days in which rats are maintained on a choline-free diet for the production of kidney damage, the kidney almost doubles in weight. The amount of water is similarly increased. The total amount of fat is increased by 28 per cent, the total amount of nitrogen by 51 per cent, but the total amount of phospholipid is diminished. If comparison is made on the 7th day (prior to the development of renal lesions) between the kidneys from the choline-deficient animals and those from the animals receiving choline, it will be noted that the weight of the kidneys is identical, the amounts of water, fat, and nitrogen are approximately the same, but the amount of phospholipid is markedly less in the choline-deficient kidneys. The ratio of phospholipid to total fat

TABLE I  
*Composition of Kidneys (Average of Twenty Animals)*

Experimental day	Treatment	Kidney weight	Moisture		Total lipid		Nitrogen		Phospholipid		Ratio total phospholipid to total lipid
			gm	per cent	total mg	per cent	total mg	per cent	total mg	total mg	
0	At weaning	0.59	78.6	463.7	2.9	17.1	2.8	16.5	2400	14.2	0.83
7	No choline	0.70	78.8	551.6	2.5	17.5	2.6	18.2	1526	10.7	0.61
	Choline	0.70	78.3	548.1	2.7	18.9	2.7	18.9	2315	16.3	0.86
10	No choline	1.00	78.8	788.0	2.2	22.0	2.5	25.0	1358	13.5	0.61
	Choline	0.78	79.4	619.3	2.7	21.1	2.7	21.1	2470	19.3	0.91

has already been appreciably diminished and this low ratio continues through the 10th day.

These results provide further evidence that kidney lesions which develop in young rats deprived of choline are consequent to a diminished supply of phospholipid. The decrease in phospholipid concentration in the kidneys of the choline-deficient rat is therefore not due to dilution of the phospholipid by an increase in the amount of some other cellular constituent.

The figures for the composition of the liver are given in Table II. Choline deficiency results in a marked increase in liver fat and a marked decrease in moisture. The weight of the liver from animals receiving choline and that from animals deprived of choline has increased during the 10 day experimental period. In the animals receiving the choline supplement the total amounts of water, fat, and phospholipid have increased in proportion to the weight of the liver. However, in the animals deprived of choline,

while the weight of the liver has increased, the total amount of phospholipid in the liver is less than the amount at the beginning of the experiment. Since the total amount of phospholipid is less in the livers of the animals deprived of choline, the concentration of phospholipid in these livers is not decreased by dilution. It will be noted that the ratio of phospholipid to total fat is very markedly reduced in the liver by choline deficiency.

TABLE II  
*Composition of Liver (Average of Twenty Animals)*

Experimental day	Treatment	Liver weight	Moisture		Total lipid		Phospholipid		Ratio total phospholipid to total lipid
			gm	per cent	total mg	per cent	total mg	mg per cent	
0	At weaning	2.27	72.8	1.65	3.5	79.5	2640	60.0	0.76
7	No choline	3.49	62.2	2.18	19.8	691.0	1450	50.8	0.07
	Choline	2.89	72.1	2.08	4.4	125.3	2405	69.6	0.55
10	No choline	3.16	64.2	2.03	20.0	632.0	1300	41.1	0.07
	Choline	3.42	72.0	2.46	3.8	130.0	2400	82.0	0.63

TABLE III  
*Weight, Phospholipid Content, and Choline Content of Liver and Kidney With and Without Choline (Average of Twenty Animals)*

Organ	Treatment	Organ weight	Phospholipid		Moisture	Choline	
			gm	mg per cent	total mg	per cent	mg per gm dry weight
Kidney	No choline	1.04	1400	14.5	78.9	9.92	209.5
	Choline	0.69	2363	16.3	78.0	13.40	294.8
Liver	No choline	3.32	1500	49.8	67.0	5.82	192.1
	Choline	3.30	2451	80.8	72.0	10.58	296.2

Estimations of the choline contents of the liver and kidneys from rats with and without choline on the 10th experimental day were made, Table III gives the results.

A deficiency of choline in the diet resulted in a 29 per cent decrease in the concentration of choline in the kidneys and a 35 per cent decrease in the choline concentration in the liver. However, the average amount of choline per 100 mg of phospholipid in the kidney of the choline-deficient animals is 15 mg, in the choline-treated animals 13 mg. This approximate similarity indicates that the amount of phospholipid formed is related directly to the amount of choline available.

## DISCUSSION

The results here given, considered in conjunction with those previously published (1, 2), provide a sequence of concordant evidence in support of the explanation of renal damage that the kidney lesions and fatty infiltration of the liver are consequent to a diminished supply of phospholipid. The several types of evidence are summarized: (1) The percentage concentration of phospholipid in the kidneys and livers of the choline-deficient animals was markedly reduced below the concentration of phospholipid in the kidneys and livers of animals kept normal by the administration of choline. (2) The decrease in phospholipid concentration of the kidney during the first 7 days on the choline-deficient diet is not due simply to dilution of the phospholipid by an increase in the amount of some other cellular constituent, since the absolute quantity is less. (3) The ratio of total phospholipid to total fat is markedly diminished prior to the development of kidney lesions. (4) The kidneys of the animals maintained on the choline-deficient diet for 10 days contain only about two-thirds as much choline calculated as per cent of dry or fresh kidney as do the kidneys from control rats receiving 3 mg of choline per gm of food. The amount of choline per 100 mg of phospholipid in the kidneys of the choline-deficient animals is approximately the same as in the choline-treated animals, indicating that the amount of phospholipid formed is related directly to the amount of choline available. (5) Triethylcholine and arsenocholine (unpublished data), when substituted for choline in the diet of the rat, prevent renal damage and fatty infiltration in the liver because they maintain the concentration of phospholipid in both these organs at a normal level. (6) The rate of phospholipid turnover in both the livers and kidneys of animals receiving choline in the diet is much more rapid than in these organs of animals deprived of choline. In normal young rats the phospholipid concentration and phospholipid turnover in the kidneys are greatest in the brief period in which a deficiency of choline causes marked renal lesions. The phospholipid turnover is markedly reduced in the livers and kidneys of older rats. This explains why the choline requirement is significantly less in rats over 35 days of age, why rats of this age are more resistant to choline deficiency, and why the renal lesions occur in young rats on a choline-deficient diet during one brief 10 day period after weaning. The decreased phospholipid turnover, and therefore the decreased requirement for choline by rats over 35 days of age, may explain the spontaneous recovery of rats which survive the crucial 10 day period, as noted by Griffith (9).

These results are in accord with those of Stetten and Grail (10) with regard to liver lipids. They may seem to differ from the data obtained by Jacobi and Baumann (11) but comparison is difficult because of differences in experimental conditions. We used a diet containing 10 per cent purified

casein and 0.5 per cent cystine, the diet used by Jacoby and Baumann contained 18 per cent crude casein and no cystine. The difference in the amount of casein, and the consequent difference in the amount of methionine available to the animals, might explain the choline synthesis observed by Jacoby and Baumann and not found by us.

#### SUMMARY

In a 10 day experimental period, during which a deprivation of choline causes kidney abnormalities in young rats, there is a reduction in the actual amount of phospholipid in the kidneys and livers as well as a decrease in the ratio of phospholipid to total lipid. There is also a decrease in the amount of choline, although the ratio of choline to total phospholipid is practically unchanged. These results, in conjunction with concordant evidence previously published, support the hypothesis that the kidney damage is due to a decreased formation of phospholipid.

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# STUDIES IN CARBOHYDRATE METABOLISM

## III METABOLIC DEFECTS IN ALLOXAN DIABETES\*

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The two current theories of pancreatic diabetes ascribe the glucosuria on the one hand to non-utilization of glucose and on the other hand to overproduction of glucose (1). The relative contributions of these two mechanisms to the urinary glucose may readily be estimated if either the dietary glucose or the glucose synthesized by the animal is isotopically labeled. In studies previously reported (2, 3) we have shown that it is possible to label that fraction of hexose that was synthesized *in vivo* by allowing such synthesis to proceed in a medium of body fluid enriched with  $D_2O$ . From these earlier experiments, in which the nature of the precursors of liver and muscle glycogen was investigated, it was possible to estimate the fraction of glycogen derived from the direct condensation of non-isotopic dietary glucose and the fraction (containing deuterium) derived by synthesis from fragments smaller than hexose. It was expected that the application of a similar technique would reveal the nature of the precursors of the urinary glucose in diabetes.

The method selected for the induction of hypoinsulinism was the administration of alloxan, which has been shown to cause a selective destruction of the cells of the islets of Langerhans (4). Its use circumvents the technical difficulties of pancreatectomy. Although the response to alloxan is less uniform in rats than in rabbits (5),<sup>1</sup> we have elected to employ rats in this study, in conformity with our earlier experiments. The diet used was nutritionally equivalent to that used before (2), and contained 60 per cent of carbohydrate, supplied in this case as starch rather than as glucose, in order to avoid contamination of the urinary glucose by spilled food.

For the isotope experiment an adult rat exhibiting a satisfactory response to alloxan was selected. The rat was fed the high carbohydrate diet throughout the experiment. During the preliminary period, following the injection of alloxan, there was a mild ketonuria, which proved transient, and a loss in body weight, notably in depot fat. The only striking per-

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<sup>1</sup> Bailey, C. C., personal communication.

sistent symptoms were severe polydipsia and polyuria together with glucosuria that fluctuated between 3 and 9 per cent 10 days after the injection of alloxan,  $D_2O$  was administered subcutaneously and in the drinking water, as in the previous experiments (2) Because insufficient allowance was made for the polydipsia, the deuterium concentration of the body water increased steadily during the course of the experiment

At frequent intervals urine was collected and analyzed for glucose The glucose was then isolated as the pentaacetate, and its deuterium concentration compared with that of the water simultaneously excreted After 56 hours of  $D_2O$  administration the rat was killed and glycogen and

TABLE I

*Isotopic Composition of Urinary Glucose*

Glucose was isolated from the urine of a diabetic rat at intervals after the  $D_2O$  level of the body fluids had been elevated The concentration of deuterium in each of the glucose samples has been compared with the simultaneous concentration in the urine water and the fraction of the glucose in the urine that had been synthesized calculated

Time	Urine volume	Glucose content	Glucose content (a)	D in urine water (b)	D in glucose (c)	D in glucose (d)*	Glucose synthesized (e)†
hrs	cc	per cent	gm	atom per cent	atom per cent	per cent of D in water	gm
4	28	4.35	1.2	1.70	0.188	11.0	0.2
22	33	8.65	2.9	1.83	0.379	20.7	1.0
30	45	4.45	2.0	2.02	0.267	13.2	0.5
48	115	4.55	5.2	2.17	0.296	13.6	1.2
56	63	4.60	2.9	2.28	0.309	13.5	0.7
Totals	284		14.2				3.6

$$*d = \frac{100c}{b}$$

$$†e = \frac{12 \times a \times d}{7 \times 100}$$

fatty acids were isolated from liver and carcass The deuterium concentrations of these samples were compared with that of the body water at the time of death

In the interpretation of the deuterium concentrations of the urinary glucose samples (Table I) it must be borne in mind that the 5 hydroxylic hydrogen atoms of glucose are immediately exchangeable on solution in water, and furthermore are certainly lost in the formation of the pentaacetate If a sample of glucose had the same concentration of deuterium as the urine water, it would, after isolation as the pentaacetate, contain no more than seven-twelfths as much deuterium as the water We have

previously presented evidence that when hexose is synthesized in a medium of heavy water from fragments such as lactate, essentially all of the hydrogen in the sugar is derived from the aqueous environment (3). If it be provisionally assumed that all the carbon-bound deuterium present in the urinary glucose was introduced by such synthetic processes, and not by simple exchange with hydrogen of the water, the fraction of urinary glucose that was synthesized *in vivo* may be calculated.

We have carried out two experiments which we believe make the possibility of exchange of carbon-bound hydrogen atoms appear unlikely. The hydrogen on the second carbon atom of glucose would, because of the ease with which enolization could occur, be suspect of such exchange. If this took place, a higher concentration of deuterium might be anticipated in this position. By converting a sample of isolated urinary glucose into its osazone, we have eliminated this hydrogen, and from the analysis of the product it could be shown that the isotopic composition of the hydrogen at the 2 position was the same as the average of the 6 remaining carbon-bound hydrogen atoms. Additional evidence that little, if any, exchange of the carbon-bound hydrogen atoms occurs in neutral solution was obtained by heating normal glucose in heavy water for a protracted period. Analysis of the pentaacetate derived from this glucose indicated that only 1 per cent of the carbon-bound hydrogen atoms had been replaced by the deuterium of the solvent.

On the basis of these results, we have calculated that of the 14.2 gm of glucose excreted during the 56 hours of observation, 3.6 gm or about 25 per cent were synthesized from fragments smaller than hexose. This synthesis proceeded in spite of the fact that the animal was ingesting quantities of sugar far in excess of its capacity for utilization. We do not interpret this as particularly favoring any theory that glucosuria in diabetes results predominantly from an excessive synthesis of glucose, as, in our rat, some 75 per cent of the urinary glucose was clearly of dietary origin. Rather do we incline to the view that gluconeogenesis proceeds in the diabetic animal much as in the normal animal and that the urinary glucose is a mixture of glucose of both dietary and of synthetic origin.

When the body fluids of normal, non-diabetic rats, on a diet equivalent to that used in the present experiment, were enriched with  $D_2O$ , deuterium was incorporated into the glycogen of liver and carcass (2). Interpolation from these data reveals that in the normal rats the deuterium concentration in the liver glycogen would reach 23, in the carcass glycogen, 9 per cent of that in the body fluids in 56 hours. Even at infinite time these values would approach 29 and 26 per cent respectively. The incorporation of deuterium into glycogen is a much more rapid process in the diabetic than in the normal animal (Table II). The actual rate of glycogen turnover in

the diabetic rat cannot be calculated from the figures presented as the values of  $z_{\max}$ , the isotope concentrations at infinite time, are not known. It is apparent, however, that the liver glycogen in the diabetic animal rapidly achieves a concentration of deuterium far in excess of that reached in the normal animal at infinite time. All of our previously reported experiments conform with the view that, whereas glycogen directly synthesized from dietary glucose in a medium of heavy water is poor in deuterium, glycogen synthesized from fragments smaller than hexose is rich in deuterium (3). The present finding of 43 per cent as high a level of deuterium in the liver glycogen as in the body water, after 56 hours, is taken to mean that in the diabetic animal a larger proportion of the liver glycogen was synthesized from fragments smaller than hexose than was the case in the normal, non-diabetic rat. Similar findings in thiamine-deficient rats

TABLE II  
*Isotopic Composition of Body Constituents*

A diabetic rat was killed 56 hours after the administration of  $D_2O$  and glycogen as well as fatty acids was isolated from liver and carcass. The deuterium concentrations of these substances have been compared with the deuterium concentration of the body water at the time of death.

	Weight	Deuterium	Deuterium
	gm	atom per cent	per cent of D in body water
Body water		2.32	100.0
Liver fatty acids	0.164	0.212	9.1
Carcass fatty acids	1.154	0.209	9.0
Liver glycogen	0.124	1.00	43.1
Carcass glycogen	0.290	0.518	22.3

(6) have previously led us to the conclusion that in this condition also glycogenesis proceeds preferentially from fragments smaller than hexose rather than directly from dietary glucose (3).

In the normal rats previously described (2) it was shown that only about 0.44 gm of the dietary glucose was used daily in the synthesis of glycogen whereas about 5 gm were used each day in the synthesis of fatty acids. It follows that only a small amount of extra glucose would result from the complete cessation of glycogenesis, whereas interference with the synthesis of fatty acids could contribute much more extra glucose. Since there was no evidence of cessation of glycogenesis in the diabetic rat, and since about 5 gm of glucose were lost in the urine daily, it is of interest to consider the rate of synthesis of fatty acids from dietary carbohydrate in the diabetic animal.

Very little depot fatty acids remained at the time of death, and the

deuterium concentration was the same as that of the fatty acids of the liver. This latter finding, which is in marked contrast to what has been found in similar experiments with normal animals (7), suggests a very efficient "mixing" of fatty acids from liver and depot. That the synthesis of fatty acids has proceeded more slowly than normal is indicated by the fact that after 56 hours the deuterium level in the fatty acids of the liver was only 9 per cent of that in the body water. In the same length of time in normal rats on the same diet (2), the corresponding value would have been 22 per cent.

On the assumption that one-half of all the hydrogen in freshly synthesized fatty acid is derived from the body fluid (8), the actual quantity of fatty acid synthesized may be estimated as  $(2 \times 9)/100 \times (0.16 + 1.15) = 0.24$  gm in 56 hours, or about 0.1 gm per day. This figure is to be compared with 1.9 gm of fatty acid synthesized from dietary carbohydrate daily by normal rats (2). *In the diabetic rat the utilization of glucose for fatty acid synthesis has been reduced to about 5 per cent of the normal figure.*

From these considerations we conclude that in diabetes, as we have previously shown to be the case in thiamine deficiency (6), there is a marked impairment in the synthesis of fatty acids from carbohydrate precursors. The loss in depot fat incident to diabetes must in good part result from this retardation of synthesis, and a large portion of the urinary glucose must be ascribed to the non-utilization of sugar, both dietary and newly formed, in the synthesis of fatty acids.

#### EXPERIMENTAL

The diet used in this experiment contained 60 per cent of corn-starch, 22 per cent of casein (Labco, vitamin-free), 6 per cent of yeast powder, 6 per cent of salt mixture (9), and 6 per cent of roughage (Celluration).

Diabetes was induced in adult male white rats of the Sherman strain by a single subcutaneous injection of a solution containing 200 mg of alloxan monohydrate per kilo of body weight. Of five rats so treated, two died in severe ketosis within 48 hours, one failed to show persistent glucosuria, and two developed the signs of chronic diabetes. To protect the rats from the early hypoglycemia (10), during the 24 hours immediately following the injection of alloxan the drinking water was replaced by 5 per cent glucose solution.

The rat selected for the isotope experiment weighed 234 gm initially. There was a rapid loss in weight during the first 8 days but during the last 5 days of the experiment the weight remained fairly constant at  $175 \pm 10$  gm. By the 5th day the urine volume had increased to over 100 cc per day, and thereafter fluctuated between 100 and 200 cc daily. The urine gave a faint test for acetone for the first 4 days but was acetone-free

thereafter At no time could the presence of acetoacetic acid be detected in the urine Glucose was determined quantitatively in the urine by the method of Folin (11) adapted to a photoelectric colorimeter The daily glucose excretion varied between 3 and 10 gm

On the 10th day, 99.5 per cent  $D_2O$  was injected subcutaneously in a quantity calculated to bring the level in the body fluids to about 1.80 per cent Simultaneously the drinking water was replaced by 2.5 per cent  $D_2O$  The animal was kept in a metabolism cage with a screen floor and urine samples were collected at the times indicated (Table I)

The samples of urine were cleared with diatomaceous earth and concentrated *in vacuo* to a thick syrup After addition of 4 volumes of acetic anhydride the mixture was stirred and heated on the steam bath until a homogeneous solution was obtained The product was again concentrated *in vacuo* and treated a second time with acetic anhydride An amount of fused sodium acetate equal to the quantity of glucose in the sample was also added After the mixture had been stirred at  $100^\circ$  for 3 hours, most of the solvent was removed by vacuum distillation and the residue stirred for 3 hours with 5 volumes of ice water This was then extracted with 5 portions of chloroform, the chloroform layers were combined, washed with 5 per cent aqueous  $Na_2CO_3$  and then with water until the washings were neutral The chloroform solution was decolorized with norit and dried over  $CaCl_2$  After the solvent had been removed *in vacuo*, the residue was dissolved in 2 volumes of boiling ethanol This solution, on being slowly cooled and seeded, deposited a crystalline precipitate, which was filtered off and washed with a little cold ethanol One recrystallization of this material from 50 volumes of boiling water gave snow-white crystals of pentaacetyl glucose The acetyl content and the reduction equivalent of this product were 98.5 and 99.0 per cent respectively of the values demanded by theory On the basis of the melting points obtained,  $118-125^\circ$ , the product appeared to be a mixture of  $\alpha$ - and  $\beta$ -glucose pentaacetates One sample was further recrystallized until a melting point of  $130^\circ$ , the value reported for pure  $\beta$ -glucose pentaacetate (12), was obtained However, as this further purification did not alter the deuterium content of the sample, and as it entailed a loss of almost half the material, it was not carried out on the remaining samples The deuterium concentration in the glucose was obtained from the analysis of the pentaacetate by multiplying the latter figure by 22/12

Isolation and analysis of carcass and liver glycogen and fatty acid samples were carried out as described previously (2)

1.5 gm of glucose pentaacetate, which contained 0.161 atom per cent deuterium, were refluxed for 3 hours with 25 cc of 0.3 N  $H_2SO_4$  To this solution were added 12 gm of sodium acetate crystals, 2.5 gm of phenylhy-

drazine hydrochloride, and 1 cc of a saturated aqueous solution of sodium bisulfite. The mixture was heated at 100° for 3 hours and then allowed to stand in the refrigerator for 48 hours. The glucosazone crystals were filtered off and once recrystallized from ethanol, m p 203° with decomposition, deuterium content, 0.142 atom per cent.

As all of the isotope in the pentaacetate was distributed among the 7 carbon-bound hydrogen atoms of the glucose, the average isotopic composition of the hydrogen atoms in these positions may be computed as  $0.161 \times 22/7 = 0.51$  atom per cent D. In six of these seven positions that were still occupied by hydrogen in the osazone, the average isotopic composition was  $0.142 \times 22/6 = 0.52$ , indicating that the isotopic composition of the H atom on carbon atom 2, that was lost during formation of the osazone, was the same as the average of the 6 carbon-bound hydrogen atoms remaining.

2 gm of normal glucose were dissolved in 10 cc of 2.5 per cent D<sub>2</sub>O and the solution sealed in an ampule. It was first autoclaved at 120° for 30 minutes and then kept at 37° for 5 days. The ampule was opened and the water distilled off at the oil pump. It contained 2.28 per cent of D<sub>2</sub>O. The residual glucose was promptly acetylated and the pentaacetate analyzed for deuterium. The product contained 0.018 atom per cent D, corresponding to 0.033 atom per cent in the hydrogen atoms of the glucose residue.

#### SUMMARY

The origin of urinary glucose in a rat suffering from alloxan diabetes has been studied with the aid of deuterium. On a 60 per cent carbohydrate diet it was found that about one-fourth of the urinary glucose was synthesized *in vivo* and about three-fourths was derived from the dietary carbohydrate.

The proportion of liver glycogen synthesized in the diabetic animal from fragments smaller than hexose was greater than had been found in normal rats.

Synthesis of fatty acids had decreased to about 5 per cent of the normal rate. The quantity of glucose not utilized as a result of this failure of fatty acid synthesis is of the same order of magnitude as that recovered in the urine. This failure of the diabetic animal to utilize glucose in the synthesis of fatty acids constitutes a major metabolic defect in this disease.

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# ON THE DETERMINATION OF ESTERIFIED CHOLESTEROL\*

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Since the ratio of total to esterified cholesterol in blood may have diagnostic importance, particularly with reference to liver damage (1-5), the desirability of a rapid and accurate method for determining these substances is obvious. Most methods now in use involve the Liebermann-Burchard color reaction but, in many cases, no account is taken of the fact that esterified cholesterol produces more color in this reaction than does free cholesterol. Such procedures, of course, give results which are too high. This error is eliminated in the methods of Schoenheimer and Sperry (6) and of Kelsey (7) by the use of a saponification procedure. However, for routine use it would be desirable to eliminate this time consuming step.

This study has shown that the color produced in the Liebermann-Burchard reaction by the esterified cholesterol of calf blood is, within certain limits, a definite percentage greater than that produced by the same amount of free cholesterol. Hence, by application of an appropriate correction factor, it is possible to determine, with reasonable accuracy, total and esterified blood cholesterol without saponification.

## EXPERIMENTAL

According to data reported by several authors (7-12) the color produced with acetic anhydride and concentrated sulfuric acid by esterified cholesterol is greater, possibly by as much as 30 per cent, than that produced by free cholesterol. Since these results, in most cases, have been arrived at by use of single esters such as the palmitate or stearate, it seemed advisable to see what average figure would result from similar determinations on blood. To do this several blood samples (calf) were analyzed for total cholesterol after saponification, and for apparent total and esterified cholesterol without saponification according to the method described later under "Procedure." Free cholesterol was calculated by difference in the latter case and is a true value because the increase in color due to the esters was the same in the determinations of both the total and esterified fractions. The free cholesterol was then subtracted from the figure obtained for total cholesterol by the saponification procedure, thus leaving the true value for esterified cholesterol. Typical values thus obtained are presented in Table

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I A series of 100 such determinations on twenty-nine calves gave an average of approximately 25 per cent more color produced by esterified cholesterol than by the same samples after saponification. The range of values was from 18 to 32 per cent.

### Procedure

The method used was essentially that of Sackett (13) except that saponification according to Kelsey's method (7) was included for those samples which were subjected to hydrolysis. The following description of the procedure brings out the modifications which were employed, particularly with respect to the esterified cholesterol determination.

*Total Cholesterol*—The determination of total cholesterol is carried out as described by Sackett (13). It is preferable that color development be

TABLE I  
*Esterified Cholesterol Determinations in Blood Plasma*

Sample No	Saponification procedure	No saponification	Difference
	mg	mg	per cent
1	110	138	25.4
2	82	107	30.5
3	107	135	26.1
4	77	97	25.9
5	95	115	21.0
6	56	68	21.4
7	54	63	25.9
8	75	95	26.7
9	88	106	20.4
10	84	107	27.4
Average			25.07

carried out in a 24° water bath for 15 minutes, at the end of which time the extent of absorption of the color developed is measured in a photoelectric colorimeter (Klett-Summerson with Filter 42). A blank of pure chloroform and standard solutions of pure cholesterol in chloroform are treated in the same manner as are the unknowns and are used for standardizing the instrument.

*Esterified Cholesterol*—Extraction, centrifuging, and evaporation are carried out exactly as for total cholesterol. After evaporation of the alcohol-ether solution, the cholesterol is transferred to a 15 ml centrifuge tube with three 3 ml portions of petroleum ether. Next is added 0.1 ml of a digitonin reagent prepared according to Leiboff (14), and the contents of the tube are thoroughly mixed by occasional inversions over a period of 10 minutes. The tubes are now centrifuged, after which the supernatant solution is decanted through a cotton plug from the precipitate of cholesterol.

digtonide This solution contains the cholesterol esters and is evaporated to dryness in a small beaker The esterified cholesterol is extracted with chloroform and then treated exactly as in the method for total cholesterol (13)

*Calculation*—Apparent total and apparent esterified cholesterol are calculated from the colorimetric readings by the usual method However, since esterified cholesterol produces on the average 125 per cent of the color produced by free cholesterol, the results obtained by the above calculation are too high by an amount equal to 20 per cent of the apparent esterified cholesterol Therefore, if the apparent total cholesterol is designated  $A$  and the apparent esterified cholesterol is designated  $B$ , true total cholesterol equals  $A - 0.20B$  and true esterified cholesterol equals  $B - 0.20B$  Free cholesterol is, of course, the difference between these two values Although the range of increased color due to bound cholesterol was found to be from 18 to 32 per cent, the error introduced by assuming a constant of 25 per cent is not great Since the calculation involves subtraction of equal amounts from both the total and esterified values, the effect of this error on the ratio of total to esterified cholesterol is, for practical purposes, insignificant

#### SUMMARY

Analysis of 100 samples of blood plasma from twenty-nine calves has shown that the esterified cholesterol of calf blood produces, on the average, approximately 25 per cent more color with the Liebermann-Burchard reagents than does the same amount of free cholesterol This fact must be considered in the calculation of results when a method is used which does not involve saponification

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# THE HISTIDINE CONTENT OF ADULT AND FETAL BOVINE HEMOGLOBIN

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The question whether the blood of the mammalian fetus contains a characteristic hemoglobin which differs in properties from the hemoglobin of the blood of its parent has assumed considerable importance during the past decade. The literature was recently thoroughly reviewed by Wyman, Rafferty, and Ingalls (1), who pointed out that the evidence that has been presented does not lead to a simple straightforward conclusion. They were able to show, however, that the solubility of carbonylhemoglobin obtained from the blood of the bovine fetus is more than 6 times as great as that of the carbonylhemoglobin from the blood of the adult cow when the measurements are made at pH 6.8 in phosphate buffers of ionic strength 4.9 to 5.5. They accordingly drew the conclusion that the two hemoglobins are distinct substances.

Further evidence has been obtained by Andersch, Wilson, and Menten (2) who examined the electrophoretic mobility of carbonylhemoglobins prepared from blood collected from the human umbilical cord, as well as from young infants, in comparison with samples derived from human adults. All of the specimens gave evidence of the presence of two components of different mobility, but whereas the component of higher mobility greatly predominated in the hemoglobin of the infant, the relative proportions were reversed in that of the adult. The mobility of the more rapidly moving component was nearly twice that of the slower. Measurements were also made of the sedimentation constants by means of a McBain type ultracentrifuge (3) and a significant difference was found, the constant for the adult hemoglobin being the greater.

These observations of differences in characteristic physical properties between the hemoglobins of the very young and of the mature animal encouraged the hope that differences in the amino acid composition might be demonstrable. Through the courtesy of Professor Wyman, samples of fetal and adult bovine hemoglobin were made available for the determination of histidine. The results of the analyses, which are given in detail in Table I, provide evidence that the proportion of histidine yielded by adult bovine hemoglobin is significantly greater than that yielded by fetal bovine hemoglobin. As has been pointed out in a recent paper (4), <sup>†</sup> maximum error to be anticipated in determinations of histidine by

dichlorobenzenesulfonate method is of the order of 3 per cent where the quantities of histidine isolated are about 100 mg. For quantities of about 150 mg, which was the case in the present analyses, the error would be expected to be appreciably less, probably not greatly in excess of 2 per cent. Only if the present determination of histidine in adult hemoglobin were 3 per cent too high and that in the fetal hemoglobin were at the same time 3 per cent too low would identity between the two proteins with respect to histidine content be at all likely. The close agreement

TABLE I

*Histidine Yielded by Bovine Hemoglobin*

The weights of recrystallized disulfonate were corrected by adding 10.2 mg per 20 ml of mother liquor

Hemo globin	Quantity of protein taken	Yield calculated from weight of bis 3 4-dichloro- benzenesulfonate				Decomposi- tion point of disulfonate	Nitrogen content of disulfonate theory 6.90
		Initial separation uncorrected for solubility	Recrystal- lized once corrected for solubility	Recrystal- lized twice corrected for solubility	Recrystallized twice corrected for solubility of initial crystals		
Adult	gm	per cent	per cent	per cent	per cent	C	per cent
	2.246	7.00	6.76	6.77	6.75	277-278.5	
		7.05	6.72	6.71	6.82	277-278.5	
		7.00	6.75	6.74	6.85	278-279	
Average		7.02	6.74	6.74	6.81 ± 0.05		6.89*
Fetal	2.217	6.67	6.35	6.33	6.45	280-281	6.97*
		6.62	6.36	6.33	6.45	279-280	
		6.66	6.36	6.32	6.44	280-281.5	
		6.65	6.37	6.36	6.48	280-281	
	2.266	6.60	6.28	6.26	6.38	278-279	6.86*
		6.51	6.26	6.25	6.36	278-280	
		6.48	6.34	6.32	6.43	279-280	
Average		6.60	6.33	6.31	6.43 ± 0.04		6.92

\* Nitrogen determined in pooled sample

between the two independent analyses of the fetal material makes it improbable that so great an error occurred.

## EXPERIMENTAL

The samples of the two types of bovine carbonylhemoglobin were prepared in the laboratory of Professor Wyman at Harvard University by the method described by Wyman, Rafferty, and Ingalls. After being crystallized, dissolved in water, and thoroughly dialyzed, each of the

solutions was mixed with an equal volume of alcohol to coagulate the protein. The coagula were thoroughly extracted with alcohol, which removed little or no color, were dehydrated with absolute alcohol, washed with ether, and dried and equilibrated with respect to moisture content by being exposed to the air for several days. The adult hemoglobin preparation contained 15.73 per cent of nitrogen, 5.46 per cent of moisture, and 0.96 per cent of ash. Corrected for moisture content and for ash in excess of 0.47 per cent, that is to say for the quantity to be expected from the 0.33 per cent of iron that this hemoglobin contains (5, 6), the nitrogen content was 16.72 per cent. The fetal hemoglobin preparation contained 15.72 per cent of nitrogen, 5.43 per cent of moisture, and 0.57 per cent of ash. Corrected in the same way, the nitrogen content was 16.64 per cent. This correction involves the assumption that the iron content of fetal bovine hemoglobin is the same as that of the adult. No information on this point was found in the literature, but in view of the extraordinary constancy in the iron content of the many varieties of hemoglobin that have been examined (for literature see (5, 6)), the assumption appeared to be justified. The corrected nitrogen contents of the two preparations are not significantly different from each other, the precision of the individual data upon which they rest being inadequate to establish this. Nevertheless the separate values found were employed in computing the quantity of protein taken for the histidine analysis.

For the determinations of histidine, about 12 gm. of each preparation were taken for hydrolysis, this being sufficient to give approximately 150 mg. of histidine in each of the aliquots analyzed. The mixture of protein and hydrochloric acid was heated for several hours on the steam bath, before being boiled, in order to overcome the tendency to froth. Aside from this minor variation, the technique of the dichlorobenzene-sulfonic acid method recently described (4) was followed in exact detail.

#### DISCUSSION

The histidine of adult bovine hemoglobin is significantly lower than that of man or of the horse or sheep (7). The available data, all of which have been obtained by the dichlorobenzenesulfonate method, are collected in Table II and are arranged in decreasing order of magnitude. The figures for histidine nitrogen in per cent of the protein nitrogen, shown in the second line of the table, are free from assumptions regarding the nitrogen and ash content of the proteins. They depend merely upon the accuracy of the Kjeldahl nitrogen determination in the hydrolysates and upon the weight and purity of the specimens of the isolated histidine compound.

The last two lines show calculations of the number of moles of histidine



per 66,700 gm of hemoglobin. This quantity, the assumed weight of a mole of hemoglobin, depends upon the iron content which, for the first four proteins, has been shown to be 0.335 per cent with uncertainty only in the third decimal place, and upon the osmotic pressure measurements of Adair (8) and the ultracentrifuge studies of Svedberg and Nichols (9). The assumption is presumably valid for the four kinds of adult hemoglobin shown but is almost certainly incorrect for the fetal bovine hemoglobin. Analogy with the case of infant human hemoglobin, the sedimentation constant of which was found to be lower than that of the adult in the study of Andersch, Wilson, and Menten, would lead one to anticipate that the molecular weight of the predominant component of fetal bovine hemoglobin is smaller than this, being possibly a submultiple of 66,700. Be this as it may, it is of interest to note that the number of moles of histidine per mole of the protein is in all cases within 1 per cent of an integral value. The figures suggest as a possibility, however, that the present determination of

TABLE II  
*Histidine of Mammalian Hemoglobins*

	Human	Horse	Sheep	Bovine adult	Bovine fetal
% of protein	8.09	7.66	7.38	6.81	6.43
N as % of protein N	12.9	12.4	11.9	11.0	10.5
Moles $\times 10^{-3}$ per gm	52.1	49.4	47.6	43.9	41.5
“ per 66,700 “	34.8	32.9	31.7	29.3	27.7
Calculated					
Nearest integer	35	33	32	29	28

histidine in adult bovine hemoglobin is an underestimate. If the correct value is one that gives 30 rather than 29 moles per molecule, the histidine content would be 7.12 per cent. Analytical studies of other preparations will be necessary to decide this point. The information now available would lead to the suggestion that analyses should be made of material that had been repeatedly recrystallized and the crystals extensively washed in order to eliminate as much as possible of the small proportion of the more soluble component that may be assumed, from the work of Wyman, Rafferty, and Ingalls and from analogy with that of Andersch, Wilson, and Menten, to be present in preparations of hemoglobin derived from the blood of the adult. Furthermore, the homogeneity of the samples examined with respect to electrophoretic mobility should be established.

Attention may be drawn to one further point. If the analytical data in Table II are taken at their face value and assumed to be accurate, the failure of the results to lead to an exactly integral value for the number of moles of histidine per mole of protein implies lack of complete homogeneity

in all of the preparations of hemoglobin examined. Contamination of the specimens of adult hemoglobin with a small proportion of hemoglobin of the fetal type, should it develop that the fetal hemoglobin of all of the species under consideration is lower in histidine than the adult type, would suffice to alter the analytical results in the manner observed.

#### SUMMARY

Determination of the histidine yielded by samples of crystallized hemoglobin derived respectively from the blood of the adult and from that of the fetus of the cow has shown with a high degree of probability that there is a difference in the amino acid composition of these proteins. Adult bovine hemoglobin yielded  $6.81 \pm 0.05$  per cent of histidine, fetal bovine hemoglobin yielded  $6.43 \pm 0.04$  per cent. The conclusion of Wyman, Rafferty, and Ingalls that these hemoglobins are distinct substances is thus supported.

Comparison of the present observations with the results of previous determinations of histidine likewise made by the dichlorobenzenesulfonate method in the hemoglobins of adult human, horse, and sheep blood shows that all of these kinds of hemoglobin differ from each other in histidine content.

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# THE CONCENTRATION OF SOME B VITAMINS IN BULL SEMEN\*

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(Received for publication, September 6, 1944)

The rôle of certain of the B vitamins in cellular metabolism has been reviewed by Rosenberg (1) Wright and associates (2) have reported on the vitamin B content of bull testes

The necessity of certain of the B vitamins in metabolism and their presence in bull testes suggest that the level of these vitamins in bull semen might give some indications of the metabolic condition of spermatozoa

Aside from the report of Lardy and Phillips (3) on the riboflavin content of a sample of bull spermatozoa, the authors have not found in the literature data on the level of B vitamins in bull semen

This investigation was conducted to determine the level of thiamine, riboflavin, pantothenic acid, and niacin in fresh whole bull semen

## Methods

The methods used in collecting the semen, counting the spermatozoa, and estimating the motility have been described elsewhere (4, 5) Ten samples of semen from four different bulls were used for the assays

The thiamine content of bull semen was determined by the fungus growth method with use of *Phycomyces blakesleeanus* as described by Hamner and his associates (6) No check was made on the samples analyzed for the possible presence of thiazole and pyrimidine which together stimulate the growth of *Phycomyces blakesleeanus* (7)

The method of Snell and Strong (8) with the test organism *Lactobacillus casei*  $\epsilon$  was used for the determination of riboflavin Acid production by the microorganism after a 3 day incubation period at 37° was measured by titration against 0.1 N NaOH with brom-thymol blue as an indicator

The assays for pantothenic acid were conducted with the same stock culture of *Lactobacillus casei*  $\epsilon$  as was employed to assay riboflavin Samples for this assay were prepared by pancreatin digestion (9) and determined according to the method of Strong and his associates (10) The 3 day acid production of the organism was measured as with the riboflavin assays

The niacin assays were carried out according to the method of Snell and Wright (11) with the use of the test organism *Lactobacillus arabinosus*

\* The authors wish to express their appreciation to I C Gunsalus of the Laboratory of Bacteriology, College of Agriculture, and the members of the Animal Nutrition Laboratory, Cornell University, for their aid and suggestions in making these assays

17-5 According to the suggestion of Isbell (12), *p*-aminobenzoic acid was added to the basal medium. The 3 day acid production of the organism was measured by titration against 0.1 N NaOH.

TABLE I

*Thiamine, Riboflavin, Pantothenic Acid, and Niacin Content of Ten Ejaculates of Bull Semen*

Sample No	Initial motility	Sperm count	Thiamine	Riboflavin	Pantothenic acid	Niacin
	<i>per cent</i>	<i>1000 per c mm</i>	<i>γ per cc</i>	<i>γ per cc</i>	<i>γ per cc.</i>	<i>γ per cc</i>
1	90	1,638	1.10	3.06	4.66	5.54
2	80	1,748	0.96	2.29	4.25	4.19
3	70	1,428	1.52	1.94	4.60	3.28
4	70	1,377	1.14	2.18	2.70	4.53
5	60	1,402	0.91	2.45	4.87	2.48
6	50	1,306	1.08	2.15	4.60	4.36
7	50	695	0.36	1.65	2.30	2.87
8	30	881	0.42	1.99	2.73	3.92
9	30	597	1.10	1.64	3.05	2.63
10	10	635	0.28	1.52	3.30	2.50
Mean	54	1,171	0.89	2.09	3.71	3.63

### Results

The results of the vitamin B assays on bovine semen are presented in Table I. Individual and mean values in terms of micrograms per cc of fresh semen are given for the ten ejaculates studied.

On a dry weight basis<sup>1</sup> the results for thiamine and riboflavin are in fairly close agreement with the reports of Wright and his associates (2) for bull testes. Niacin and pantothenic acid concentrations in bull semen on a dry basis are much lower than reported for testes.

The spermatozoa count showed a significant positive correlation with each of the four vitamins determined. This fact suggests that the vitamins were mainly present within the sperm cells.

With the exception of pantothenic acid the vitamins determined were significantly correlated with the initial motility exhibited by the spermatozoa. This would seem to correspond with the metabolic activity concerned in promoting the motility of spermatozoa. Thus, not only sperm numbers were concerned in the variation in the concentration of these three vitamins, but the relative activity of the spermatozoa also was greater in the presence of increased quantities of thiamine, riboflavin, and niacin.

<sup>1</sup> Bull semen contains  $10 \pm 1.8$  per cent dry matter.

As would seem probable from their close relationship concerned with enzyme systems in metabolism, riboflavin content and niacin concentration showed a significant positive correlation. Thiamine and riboflavin were also associated with the trend in pantothenic acid concentration.

#### SUMMARY

The thiamine, riboflavin, pantothenic acid, and niacin content of fresh bull semen were found to be 0.89, 2.09, 3.71, and 3.63  $\gamma$  per cc, respectively.

The spermatozoa count was correlated with the concentration of all four vitamins.

Initial sperm motility was correlated with the concentrations of thiamine, riboflavin, and niacin.

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## THE ESTIMATION OF METHIONINE IN PROTEIN HYDROLYSATES AND HUMAN URINE\*

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In our efforts to characterize human amino acid deficiencies by variations in the composition of the urine, it became necessary to find a satisfactory method for the estimation of methionine in the urine. Except for the procedure suggested for protein hydrolysates by Toennies and coworkers (1), based on the selective oxidation of methionine by hydrogen peroxide in the presence of perchloric acid, none of the other available methods seemed adaptable to our purposes. Initial experiments with the oxidation method confirmed the findings of Toennies relative to the participation of cystine and tryptophane in the reaction (2). However, after a detailed study of this procedure with amino acid mixtures and protein hydrolysates we found that by suitable adjustment of the concentration of perchloric acid and hydrogen peroxide in the reaction mixture the interference from both cystine and tryptophane could be completely obviated.

The adequacy of the new method was ascertained by recovery tests and the analysis of a number of proteins. From the practical view-point it is significant that the estimation can be performed in the presence of such concentrations of HCl as occur in the usual hydrochloric acid hydrolysates of the proteins. The applicability of the procedure to the urine was established by means of a study which demonstrated that the normal urinary constituents failed to react as methionine. This practice was further justified by the observation that the ingestion of *dl*-methionine by normal individuals resulted in a prompt elevation of the methionine level in the urine as determined by our method. From a limited series of observations, it has been found that the normal adult (male) on a normal diet excretes 247 to 494 mg. of methionine daily.

### EXPERIMENTAL

#### Reagents<sup>1</sup>—

Oxidizing mixture. 96 cc. of 60 per cent perchloric acid and 4 cc. of 30 per cent hydrogen peroxide (superoxol) are mixed and made up to 300 cc. This mixture was found to keep well in the ice box for 2 or 3 months.

\* Aided by grants from the Rockefeller Foundation and the Nutrition Foundation, Inc.

<sup>1</sup> Merck "reagent" grade chemicals are used throughout.



**Catalyzing mixture** 1 gm of potassium iodide is dissolved in 100 cc of 0.1 per cent ammonium molybdate. This solution must be prepared immediately before being used.

**Sodium thiosulfate** 49.6 gm (0.1 N) are dissolved in 200 cc of water, and the solution is made up to 2 liters after the addition of 20 cc of amyl alcohol. The amyl alcohol serves as a stabilizing agent.

**Potassium iodate standard** 3.567 gm (0.1 N) of potassium iodate are dried at 110° for 1 hour and dissolved in 1 liter of water. This solution is used to standardize the thiosulfate solution.

**Starch indicator** 1 gm of Lintner soluble starch is dissolved in 100 cc of saturated NaCl solution by heating on the steam bath.

**Methionine standard** 0.5 to 0.7 gm of *dl*-methionine (9.39 per cent N found) is weighed accurately and dissolved in 100 cc of water. The solution is stored in the ice box.

### *Analytical Procedure*

**Proteins**—Hydrolysates are prepared by refluxing 50 gm of proteins with 250 cc of constant boiling hydrochloric acid for 24 hours. The total nitrogen content of the hydrolysate is determined directly by micro-Kjeldahl analysis (3), then the excess of acid is removed by concentration *in vacuo* and the humin separated by filtration. Sulfuric acid digests are prepared by refluxing 50 gm of protein with 250 cc of 25 per cent sulfuric acid for 24 hours and removing the acid as calcium sulfate. Aliquots of the hydrolysates not greater than 17 cc and containing 5 to 40 mg of methionine are measured into 250 cc ground glass-stoppered Erlenmeyer flasks. If necessary the volume of the sample is adjusted to 17 cc and 3 cc of the oxidizing mixture are added to the reaction flask. In order to allow for a reaction time of exactly 1 hour for each determination when a series of assays is being carried out, the addition of the oxidizing mixture to each specimen is spaced at 5 minute intervals, so that the time necessary for the final titration will not curtail the requisite reaction period. At the end of the hour, 30 cc of water and 20 cc of the catalyzing reagent are added to each flask of the series in the same sequence as the addition of the oxidizing mixture and the samples are titrated immediately with 0.1 N sodium thiosulfate from a 10 cc micro burette. The starch indicator solution is added after the appearance of a deep amber color. The end-point is definite but the solutions may turn blue after about 5 minutes. 1 or 2 cc of methionine standard diluted to 17 cc and an equal volume of water in separate flasks are treated similarly and simultaneously with the unknown samples to obtain the methionine factor and reagent blank respectively for the run. When the determinations are performed at 20–25°, the reagents exhibit only slight variations in these constants over a period of 2 or 3 months.

Analyses of protein hydrolysates prepared by sulfuric acid or enzymatic digestion techniques show that the method can be used on these preparations without further modification

*Human Urine*—24 hour specimens are collected in brown bottles containing 50 cc of 15 per cent HCl (by volume) and 1 cc of 10 per cent alcoholic thymol and are made to a uniform volume of 2 liters before removal of the samples for the methionine determination. It has been found that under these conditions the methionine content of the specimens remains unchanged after storage of 1 week at room temperature

Owing to the small amounts of methionine usually present in the urine, a satisfactory analysis can be performed only after the methionine concentration is augmented either by evaporation of the sample to one-fifth of its original volume or the addition of a known amount of methionine to the sample. Although the latter technique has been found more convenient and sufficiently accurate for routine purposes and is described here, the concentration technique is recommended for exact work. To 15 cc of urine in 250 cc ground glass-stoppered Erlenmeyer flasks are added 2 cc of the methionine standard followed by the addition of 3 cc of the oxidizing reagent. The preparation of the methionine standard (2 cc), water blank, and titration of the samples are carried out as already described for the proteins

#### Calculations—

$A =$  cc 0.1 N sodium thiosulfate required for water blank  
 $B =$  " 0.1 " " " " " methionine standard  
 $C =$  " 0.1 " " " " " unknown  
 $B' = A - B =$  corrected titer of methionine standard  
 $C' = A - C =$  " " " unknown  
 $F = (\text{mg methionine in standard})/B'$

Then for *proteins*, mg of methionine in sample =  $C' \times F$ , for *urine*, mg of methionine for 24 hours =  $((C' - B') \times F \times \text{total volume (2000 cc)}) / 15$

#### Results

Representative data of experiments performed to determine the concentrations of perchloric acid and hydrogen peroxide necessary in the final reaction mixture to reduce the interference from cystine and tryptophane to a minimum are shown in Table I. Apparently Mixture II is the one best suited for analytical purposes. It is evident that although the specificity of the reaction is only slightly impaired by variations in the concentration of perchloric acid, it is seriously affected by changes in the concentration of hydrogen peroxide. The other conditions of the reaction (volume, time, and temperature) were established as optimal from a large series of experiments and representative data are given in Table II. As might be expected from

TABLE I

*Effect of Variations in Concentration of  $H_2O_2$  and  $HClO_4$  on Peroxide Oxidation of Methionine, Cystine, and Tryptophane*

Mixture No	Molarity of oxidizing reagents in 20 cc mixture		Methionine 8.80mg	Cystine 1.0 mg	Tryptophane 7.0 mg	Mixture of methionine 8.8 mg cystine 7 mg tryptophane 7 mg
	HClO <sub>4</sub>	H <sub>2</sub> O <sub>2</sub>	Thiosulfate titer as methionine mg			
I	0.50	0.012	8.80	0.33	0.00	8.90
II	0.50	0.025	8.80	0.02	0.03	8.85
III	0.50	0.050	8.80	0.41	0.97	9.20
IV	0.50	0.100	8.80	4.44	3.23	16.20
V	0.25	0.025	8.80	0.00	0.16	8.98
II	0.50	0.025	8.80	0.00	0.00	8.82
VI	1.00	0.025	8.80	0.00	0.46	9.10
VII	2.00	0.025	8.80	0.00	0.62	9.22

TABLE II

*Effect of Variations of Volume, Temperature, and Time on Specificity of Peroxide Oxidation of Methionine*

3 cc of oxidizing mixture were used for each test

Reaction conditions			Methionine 6.98 mg	Mixture of methionine 6.98 mg cystine 6.85 mg tryptophane 5.32 mg	Casein digest (HCl) 63.56 mg N
Volume	Temperature*	Time	Thiosulfate titer as methionine		
cc	C	min	mg	mg	mg
10	23	60	9.16	9.58	16.70
15	23	60	7.98	8.96	17.15
20	23	60	6.98	7.05	13.55
25	23	60	5.58	6.58	13.15
30	23	60	6.18	5.38	10.15
20	15	60	5.50	5.58	13.05
20	20	60	7.00	6.98	13.45
20	25	60	6.98	7.00	13.50
20	30	60	7.08	8.00	15.80
20	35	60	7.64	8.17	15.45
20	23	30	5.38	5.38	12.35
20	23	40	6.58	6.58	13.35
20	23	60	6.98	7.03	13.55
20	23	90	7.58	7.78	14.55
20	23	120	8.58	8.98	17.55
20	23	180	8.78	10.75	17.55

\* Constant to  $\pm 1^\circ$

the above experiments, these data reveal that the accuracy of the determination is seriously affected by variations in reaction volumes. It also appears that, although the oxidation of methionine itself does not exhibit any appre-

erable temperature coefficient in the 20–30° range, considerable interference from the other amino acids is sustained above 25°. The experiments on the effect of variations in reaction time show that prolongation of the oxidation beyond the 1 hour period introduces an "overoxidation error" equivalent to +0.02 mg of methionine per minute when methionine is tested singly and +0.03 mg per minute in mixtures. Since a reagent blank is run with each set of determinations, slight variations in the reaction

TABLE III

*Effect of Addition of Various Substances on Methionine Value of Casein Hydrolysate As Determined by Peroxide Oxidation Method*

5 cc of casein hydrolysate, found to contain 28.9 mg of methionine, were used for each test

Substance*	Amount added	Thio-sul-fate titer as methionine	Error due to added substance	Substance*	Amount added	Thio-sul-fate titer as methionine	Error due to added substance
	mg	mg	per cent		mg	mg	per cent
dl-Methionine	8.9	38.0	+0.5	Thiourea	20.0	49.9	+73.0
l(-)-Cystine	8.4	29.1	+0.6	Thiouracil	25.0	153.6	+435.0
l(-)-Cysteine	8.6	29.0	+0.3	Phenylthiourea	25.0	139.0	+380.0
dl-Tryptophane	8.9	28.7	-0.6	Sulfanilic acid	25.0	28.9	0
l(-)-Tyrosine	9.6	28.8	-0.3	Sulfosalicylic acid	25.0	28.8	-0.3
l(+)-Histidine	8.6	28.9	0	Sulfanilamide	24.0	29.0	+0.3
Uric acid	27.9	28.9	0	Sulfaguanidine	25.0	29.1	+0.6
Creatine	49.9	28.8	-0.3	Sulfadiazine	25.0	29.1	+0.6
Creatinine	52.5	28.8	-0.3	Sulfathiazole	25.0	29.1	+0.6
Urea	56.0	29.0	+0.3	Thiamine chloride	25.0	28.8	-0.3
Ammonium sulfate	70.9	28.9	0	Saccharin	26.0	28.7	-0.6
Taurine	4.0	28.7	-0.6	Penicillin	1000	29.2	+0.8
Sodium taurocholate	25.0	28.8	-0.3		units		
				Glucose	100	28.9	0

\* Chemically pure compounds were used for all of these tests

conditions are permissible without seriously affecting the accuracy of the results

The adaptability of the reaction to the estimation of methionine in protein hydrolysates is demonstrated by the finding that the original methionine value of a casein hydrolysate is not significantly affected by the addition of the other amino acids tried, whereas added methionine is quantitatively recovered (Table III). The possibility of applying the reaction to the determination of methionine in the urine is indicated by the fact that the addition of the common nitrogen- and sulfur-bearing constituents of the urine also failed to alter the methionine value of the hydrolysate (Table III). A number of substances possessing sulfur groupings of theoretical

interest and others which might occur in the urine as the result of medication were also tested (Table III). The presence of large amounts of many of these compounds in the reaction mixture produced only negligible errors in the final results. Errors caused in the methionine titer of the hydrolysate by the presence of thiourea, thiouracil, and phenylthiourea indicate that the thiocarbonyl group is also susceptible to peroxide oxidation. The failure of thiazole (cyclic thio ether) derivatives to consume hydrogen peroxide is surprising in view of the behavior of methionine.

Results of the methionine analyses of some proteins are reported in Table IV. Since the presence of hydrochloric acid does not appear to alter the

TABLE IV  
*Methionine Content of Hydrolysates of Some Biological Substances*

Biological product	Hydrolyzing agent	Uncorrected N content	Moisture content	Ash content	Corrected N	Methionine N of total N	Methionine per 100 gm product
		per cent	per cent	per cent	per cent	per cent	gm
Casein, Harris*	HCl	13.62	6.80	0.38	14.63	1.96	2.85
Lactalbumin, Harris	"	11.70	7.00	1.92	12.71	1.87	2.32
Gelatin, U S P	"	14.30	12.10	0.42	16.09	0.74	1.13
Fibrin, Wilson	"	15.10	4.03	0.21	15.70	2.06	3.32
Casein, Sheffield	H <sub>2</sub> SO <sub>4</sub>	12.50				2.08	2.76
Deaminized casein	"	12.40				2.35	3.12
Oxycasein	"	12.45				0.16	0.21
Amigen	Enzymes	11.80				1.98	2.50
Human hemoglobin	HCl	13.96				1.71	2.54
" hair*	"	10.15				1.40	1.52

\* The methionine value was not altered by neutralization of the acid hydrolysate with NaOH.

methionine value found for casein, most of the analyses were performed on hydrolysates prepared with hydrochloric acid. Commercially available proteins, casein, lactalbumin, gelatin, and fibrin, were used without further refinement of the products. However, the moisture and ash contents of these products were determined and the results of the analyses corrected for these impurities. Deaminized casein was prepared by the procedure of Dunn and Lewis (4). Human hemoglobin was derived from red cells by the method of Zinoffsky (5). Oxycasein was prepared by the method described by one of us (6). Heterogeneous black hair obtained from barber shop clippings was used for this study. Enzymatically digested casein (amigen) containing 64.8 per cent free amino N was analyzed without further hydrolysis. The methionine content found for these proteins is in good agreement with those available in the literature (7).

Owing to the small amounts of methionine usually present in human urine and the relatively large amounts requisite for this method, an adequate

TABLE V  
Estimation of Methionine in Normal Urine

Sample	Methionine added	Methionine found	Methionine in urine	Recovery of added methionine
	mg	mg	mg per cc	per cent
90 cc Urine A concentrated <i>in vacuo</i> to 15 cc	0	12 00	0 134	99 4
90 " " " " " " " 15 "	13 84	25 70	0 132	
15 " " " " " " " 15 "	13 84	15 90	0 137*	
90 " " B concentrated <i>in vacuo</i> to 15 cc	0	21 10	0 235	100 6
90 " " " " " " " 15 "	13 84	35 00	0 236	
15 " " " " " " " 15 "	13 84	17 29	0 232*	

\* Calculated from (methionine found) - (methionine added)/15

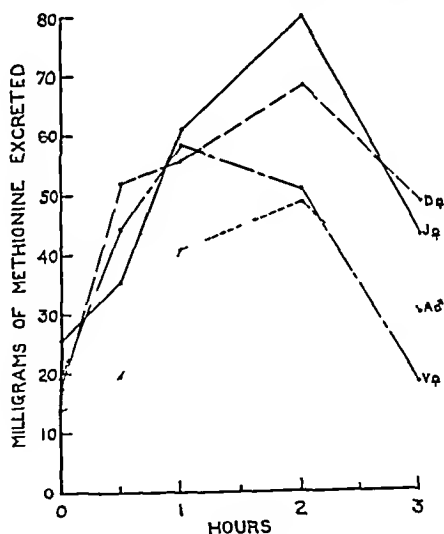


FIG 1 Excretion of methionine following ingestion of 1.5 gm of *dl* methionine by normal adult humans

determination could not be performed without augmentation of the normal methionine concentration of the urine. A series of experiments indicated that satisfactory results could be obtained when the analysis was performed (a) on a 15 cc sample derived by the 5- or 6-fold concentration *in vacuo* of

the original specimen, or (b) on an aliquot of the original specimen to which a known amount (7 to 15 mg) of methionine had been added. As shown by representative data in Table V, the results obtained by the latter device compare favorably with those derived from the more tedious concentration technique and are satisfactory for routine purposes.

Although the quantitative recovery of methionine added to the urine (Table V) points to the suitability of the method for the estimation of methionine in the urine, it was decided to secure further evidence of the specificity of the reaction from excretion tests. For this purpose four fasting normal subjects were fed 1.5 gm (0.01 M) of *dl*-methionine 2 hours after breakfast and given 240 cc of water at zero hour and 120 cc more at the end of the 1st and 2nd hours to maintain a uniform flow of urine for the period of the experiment. The urine was collected at 0,  $\frac{1}{2}$ , 1, 2, and 3 hours

TABLE VI  
*Relation of Methionine Excretion to Total N and Amino N Output in Normal Adult Male*

Subject	Body weight	Total N	Amino N	Methionine	Methionine N of amino N
	kg	gm	mg	mg	per cent
Br	103.0	19.4	596	285	5.4
Vo	94.0	17.4	696	494	6.7
Ba	74.4	15.8	459	256	5.2
Yo	81.6	10.8	392	304	7.3
So	90.1	14.3	493	313	5.9
Me	80.7	15.5	408	266	6.1
Sh	68.0	13.8	459	247	5.0

and was submitted immediately to the analyses. The rise in the urinary methionine level observed in all the subjects (Fig. 1) leaves little doubt that the reaction is specific for methionine.

The results of the methionine analysis of 24 hour urine specimens of seven normal males on normal diets are recorded in Table VI. The amino N (8) and total N (3) analyses were also performed and it appears from this limited series that the methionine N constitutes approximately  $6 \pm 1$  per cent of the total amino N.

#### DISCUSSION

As shown by the data in Table VII, the method described here for the estimation of methionine yields results which compare favorably with those obtained by the Baernstein procedure (9) or the periodide method of Lavine (10). However, the comparative simplicity and rapidity of operation of

our procedure recommend it for studies in which numerous methionine estimations of proteins or urine are required

A study of a number of compounds bearing a variety of sulfur linkages demonstrated that only terminal thio ether groups as in methionine and the thiocarbonyl group of thioureas consume hydrogen peroxide under the conditions of the reaction. This finding suggests the use of the test for the identification of these linkages in substances of unknown structure. For example, the failure of penicillin to consume hydrogen peroxide points to the absence of these two groups in the chemical structure of this product.

We are not aware of any previous assays of urinary methionine. Although the daily excretion of 200 to 500 mg of methionine by the adult

TABLE VII

*Comparison of Results of Methionine Analyses of Proteins by Peroxide Method and Other Methods*

The methionine content is given in gm per 100 gm of protein

Protein	Methods		
	Peroxide	Hydrogen iodide (Baerstein (9))	Periodide (Lavine (10))
Casein, Harris	2.85	3.10-3.31	2.85-2.89
Lactalbumin, Harris	2.32	2.32-2.45	2.35-2.41

human may appear unusual at first glance, it must be recalled that this constitutes only 5 to 7 per cent of the total daily amino N.

#### SUMMARY

A method for the rapid and convenient determination of methionine in protein hydrolysates and the urine, based on the hydrogen peroxide oxidation of methionine, is described. A limited series of observations reveals that the normal adult excretes 247 to 494 mg of methionine daily. The use of the oxidation reaction for the characterization of sulfur compounds is discussed.

We wish to thank Charlotte N. Kajdi for some experiments performed in connection with this work.

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## MANOMETRIC DETERMINATION OF FORMIC ACID

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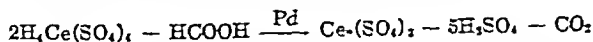
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The quantitative determination of formic acid in distillates from fermentation liquors, urine, and blood is usually accomplished by oxidizing the sample with an excess of mercuric chloride, the resulting calomel then being determined gravimetrically (1), or volumetrically by oxidation with excess iodine (2). Formic acid has also been determined colorimetrically as the aldehyde after reduction with magnesium (3-5), and manometrically by enzymatic decomposition (6, 7).

None of the above procedures is suitable for the routine determination of small amounts of formic acid in fermentation liquors. Oxidation with mercuric chloride requires a minimum of approximately 5 mg. for quantitative recoveries (2). Furthermore, both the chloride (4) and the oxide (8) of mercury are reduced by pyruvic acid, a compound relatively volatile in steam, which may cause serious errors in formic acid determinations on distillates from bacterial cultures or suspensions. The colorimetric determination of formic acid (3-5) should be relatively specific and quite sensitive, but in our hands this procedure failed to yield satisfactory and consistent recoveries. The manometric procedures for determining formic acid by enzymatic decomposition to carbon dioxide and hydrogen or carbon dioxide and water (6, 7) and by mercuric chloride oxidation to carbon dioxide and water (9) have the advantage that relatively small samples may be employed, but the former method, to be specific for formic acid, requires the preparation of a rather labile enzyme, while the latter method cannot be used with samples containing pyruvic acid.

We have found ceric sulfate and palladium to be much more satisfactory reagents for this determination, the reaction being



The  $\text{CO}_2$  liberated is easily determined by carrying out the reaction in the Warburg manometric apparatus. Since both the oxidant and acidified sample are placed in the main well of the Warburg vessel, traces of lactic and pyruvic acids are oxidized to acetic acid and carbon dioxide before the catalyst is tipped in from the side arm. The time required for the oxidation is considerably less than that required for the oxidation with mercuric chloride, and the stock reagents appear to be stable for at least 6 months.

## EXPERIMENTAL

*Reagents*—Freshly prepared solutions of ceric sulfate give large blanks in Warburg vessels containing catalyst and no formic acid. This was found to be due to oxidation of water with liberation of molecular oxygen, as suggested by Smith,<sup>1</sup> a reaction which has been shown (10) to proceed slowly in the absence of a catalyst with perchloratocerate as the oxidant. The rate of this reaction decreases sharply when there is a concomitant reduction of ceric sulfate by formic acid. Therefore a correction factor for evolved oxygen, calculated from a control vessel containing only cerate and water, cannot be applied exactly to an experimental vessel containing cerate, water, and formic acid. This difficulty has been met by poisoning the potential of the cerium sulfate at an initial ( $\text{Ce}^{4+}$ ) ( $\text{Ce}^{3+}$ ) ratio of 4:1, *i.e.*, by using a solution which is 80 per cent in respect to  $\text{Ce}^{4+}$ . The drop in potential of this solution, when used under the experimental conditions described below, falls on the straight line portion of the electrode potential- ( $\text{Ce}^{4+}$ ) curve (11), and the rate of oxidation of water is, therefore, nearly the same in both control and experimental vessels.

The following stock reagents are needed for the determination of formic acid

1 10 N  $\text{H}_2\text{SO}_4$ , 100 ml

2 10 per cent ceric sulfate in N  $\text{H}_2\text{SO}_4$ , 100 ml. Dissolve 10 gm of reagent grade ammonium tetrasulfatocerate<sup>2</sup> in 100 ml of normal sulfuric acid. Transfer 20 ml of this solution to another vessel and add 30 per cent hydrogen peroxide until the ceric sulfate is just decolorized. 2 drops of 0.002 M "ferroin" may be used as an internal indicator, although we have found this unnecessary, since the solution itself undergoes a readily apparent change from yellow to colorless upon complete reduction of the ceric sulfate. Approximately 0.3 ml of peroxide is required. Mix this cerous sulfate with the remaining 80 ml of 10 per cent ceric sulfate and store in a glass-stoppered bottle.

3 10 per cent palladinized asbestos in 0.1 N  $\text{H}_2\text{SO}_4$ , 10 ml. The palladinized asbestos (10 per cent in palladium) must be free of oxidizable compounds and well pulverized before it is used. Place several gm of the dry catalyst in a mortar and reduce to a fine powder. Suspend 1 gm of the powder in 20 to 30 ml of distilled water, make slightly alkaline with sodium carbonate, and centrifuge. Discard the supernatant fluid and resuspend the catalyst in dilute sodium carbonate. Centrifuge, suspend the catalyst in 20 to 30 ml of 0.1 N  $\text{H}_2\text{SO}_4$ , and add 0.5 ml of the ceric sulfate solution. Centrifuge, wash once with 0.1 N  $\text{H}_2\text{SO}_4$ , and finally make the catalyst up to a 10 per cent suspension by adding 0.1 N  $\text{H}_2\text{SO}_4$  to make 10 ml.

<sup>1</sup> Smith, G. F., personal communication (1943, 1944).

<sup>2</sup> Obtainable from the G. Frederick Smith Chemical Company, Columbus, Ohio.

*Determination of Formic Acid in Pure Solution*—Reagent grade sodium formate was dried 2 hours at  $100^{\circ}$ , cooled in a vacuum desiccator, and made up as a stock 0.1 N solution in distilled water. Gravimetric assay of such solutions gave 100.9 per cent recoveries with the mercuric chloride reagent of Riesser (2), and 100.0 per cent recoveries with the mercuric chloride reagent of Dakin, Janney, and Wakeman (1). The volumetric procedure described by Riesser (2) did not give satisfactory recoveries of formic acid. The recoveries which were obtained by manometric oxidation with ceric sulfate are given in Table I. For these experiments, the stock solution of sodium formate was diluted with water and 10 N  $\text{H}_2\text{SO}_4$  to contain 1 to 16 micromoles of formic acid (22 to 358 microliters of  $\text{CO}_2$ ) per ml. of N  $\text{H}_2\text{SO}_4$ . Each Warburg vessel in these determinations received 1 or 2 ml. of ceric sulfate, 1 ml. of sample in N  $\text{H}_2\text{SO}_4$ , and 0.2 ml. of catalyst. The manometer readings were followed, after the catalyst was tipped in from the side arms, until the rate of excursion of the experimental vessels had fallen to that of the control. Readings should not be continued more than 20 to 30 minutes beyond this time, since the rate of oxygen production in the control vessel is then slightly greater than that in the experimental vessels. The usual solubility constants and equations (cf. Dixon (12)) were employed in computing the  $\text{CO}_2$  production in the experimental vessels. Preliminary experiments, with a standard solution of  $\text{KHCO}_3$ , indicated that the solubility constants for  $\text{CO}_2$  in the ceric sulfate did not differ significantly from those in dilute sulfuric acid. It may be noted here that if a bank of vessels be used which differ but slightly in volume, then the control vessel can serve also as a thermobarometer and corrections for oxygen production need not be made.

The corrections for oxygen production were greater, and the recoveries of less than 10 micromoles of formic acid were less uniform when 2 ml. of ceric sulfate were used. The average recovery from thirty samples containing 10 to 15 micromoles each, to oxidize which 1 ml. of ceric sulfate was used, was 96.0 per cent. The figures given in the last column of Table I were obtained by correcting the per cent recoveries by  $100/96$ , or 1.04. The consistent recovery of only  $96 \pm 2$  per cent of the theoretical amount of  $\text{CO}_2$  from samples containing 10 to 15 micromoles of formic acid was apparently due to the production of a smaller amount of oxygen in the experimental vessels than in the control vessel. This in turn was referable to the lowered electrode potential of the ceric sulfate in the experimental vessels. Evidence to support this conclusion was obtained in two ways. (1) An amount of  $\text{CO}_2$ ,  $100 \pm 0.5$  per cent of the theoretical, was recovered in the manometric oxidation of oxalic acid by ceric sulfate in the absence of catalyst. The oxygen production in the control vessel, under these conditions, was negligible. When palladium was added to both control and experi-

mental vessels, the recoveries were  $96 \pm 0.5$  per cent and 10 microliters of oxygen were obtained in the control vessel (2) Recoveries of formic

TABLE I  
*Recovery of Formic Acid (Expressed As Microliters of CO<sub>2</sub>) in Manometric Determination by Oxidation with Ceric Sulfate*

Oxidant	Temperature	Formic acid				Oxidant	Temperature	Formic acid			
		Taken	Found					Taken	Found		
ml	°C	micro-liters	micro-liters	per cent	per cent	ml	C	micro-liters	micro-liters	per cent	per cent*
2	40	336	320	95.3	99.1	1	35	336	328	97.6	101.6
2	40	336	321	95.5	99.4	1	35	336	330	98.2	102.1
2	40	336	319	95.0	98.8	1	35	336	329	98.0	101.9
2	40	336	321	95.5	99.4	1	35	269	257	95.5	99.4
2	35	269	261	97.0	100.9	1	35	224	219	97.8	101.8
2	35	224	215	96.0	99.8	1	35	224	215	96.0	99.8
2	35	224	214	95.5	99.4	1	35	224	216	96.5	100.4
2	35	224	211	94.2	98.0	1	35	224	214	95.5	99.4
2	35	224	210	93.8	97.5	1	35	224	215	96.0	99.8
1	40	358	348	97.2	101.0	1	35	224	217	96.8	100.7
1	40	336	320	95.3	99.1	1	35	224	217	96.8	100.7
1	40	336	317	94.4	98.1	1	35	224	213	95.0	98.8
1	40	336	315	93.8	97.5	1	35	224	214	95.5	99.4
1	40	314	305	97.2	101.0	1	35	224	215	96.0	99.8
1	40	269	255	94.8	98.5	1	35	224	216	96.5	100.4
1	40	224	214	95.5	99.4	1	35	224	217	96.8	100.7
1	40	224	210	93.8	97.5	1	35	224	217	96.8	100.7
1	40	224	214	95.5	99.4	1	35	202	191	94.5	98.3
1	40	224	210	93.8	97.5	1	35	179	176	98.3	102.2
1	40	134	130	97.0	100.9	1	35	168	162	96.5	100.4
1	40	112	109	97.3	101.2	1	35	168	167	99.4	103.4
1	40	89	83	93.3	97.0	1	35	134	134	100.0	104.0
1	40	45	44	97.8	101.8	1	35	112	106	94.6	98.5
1	40	22	26	118.1	123.0	1	35	112	109	97.3	101.2
1	35	336	323	96.1	100.0	1	35	90	86	95.5	99.4
1	35	336	322	95.9	99.7	1	35	67	61	91.0	94.8
1	35	336	320	95.3	99.1	1	35	45	49	108.9	113.2
1	35	336	324	96.4	100.2	1	35	39	31	79.5	82.7
1	35	336	324	96.4	100.2	1	35	22	22	100.0	104.0
Average recovery, corrected											100.2

\* Corrected by the factor 1.04 (see the text)

acid of  $100 \pm 1$  per cent were obtained when the initial electrode potential of the ceric sulfate in the control vessel was lowered, by adding a calculated amount of oxalate, to that final potential existing in the experimental vessels

*Specificity of Method*—A number of compounds, most of which are known to occur frequently in bacterial cultures, were tested for interference in the recovery of formic acid from steam distillates. The list of compounds taken for test was intended to include several which might decompose during steam distillation, as well as those which might be slightly to readily volatile in steam and which were known (10) to yield formic acid when oxidized by ceric sulfate. For these tests, 400 to 500 micromoles of each

TABLE II  
*Compounds Tested for Interference in Determination of Formic Acid*

Do not interfere	May interfere
Acetic acid	Cinnamic acid*
Acetoin*	Ethylene glycol*
<i>p</i> -Aminobenzoic acid	Glycerol*
Benzoic acid	Glycolic acid
2,3-Butylene glycol*	$\beta$ -Hydroxybutyric acid
Isobutyric acid	$\beta$ -Hydroxypropionic acid
<i>n</i> -Butyric "	Lactic acid
<i>n</i> -Caproic "	Levulinic acid*
<i>n</i> -Caprylic "	Propylene glycol*
Citric acid	Pyruvic acid*
Diacetyl*	Salicylic acid*
Fumaric acid	
Glucose	
Glutaric acid	
Malic acid	
Malonic acid	
Oxalic "	
Propionic acid	
Serine	
Succinic acid	
Tartaric "	
Valeric "	

\* Significantly volatile in steam, and oxidized by  $\text{HgCl}_2$ .

compound were placed in a one piece flask of the Pozzi-Escot type (13), the contents were made definitely acid to brom-phenol blue by addition of  $\text{N H}_3\text{PO}_4$ , and steam-distilled. A total of 500 ml of distillate was collected. The distillate was neutralized, taken to dryness on a steam bath, and finally made up to 10 ml in  $\text{N H}_2\text{SO}_4$ . 1 ml aliquots of this were taken for manometric oxidation by ceric sulfate, as described above. The results obtained from these tests are summarized in Table II. The compounds which do not interfere fall roughly into three groups (1) those

not volatile in steam (*e g*, sugars<sup>3</sup>, dicarboxylic acids), (2) those volatile in steam but removed on the steam bath (acetoin, diacetyl), and (3) those volatile in steam but not oxidized by ceric sulfate in the presence of palladium (the saturated fatty acids)

In general, compounds which may interfere, in samples taken for oxidation, are (1) those such as lactic and pyruvic acids which may be present in appreciable concentration in steam distillates from bacterial cultures and which may, therefore, reduce an undue amount of ceric sulfate, (2) compounds which, although but feebly volatile in steam, yield formic acid when oxidized by ceric sulfate, or are oxidized at an appreciable rate by ceric sulfate only in the presence of the catalyst. From tables of distillation constants (14) and type reactions of organic compounds with ceric sulfate (10), it was apparent that cinnamic, glycolic, and levulinic acids might interfere in this determination. Further examination indicated, however, that these acids could be effectively removed by taking the first distillate to dryness, redissolving, and redistilling (Table III). Redistillation did not appear to be necessary with samples containing relatively small amounts of levulinic acid or glycerol, since only a trace of these compounds appeared in the first distillate. Similarly, ethylene and propylene glycol need not interfere seriously, because only a trace of these appears in the distillate, and this may be removed by holding the neutralized and dried distillate at 100° for 1 to 2 hours. Cinnamic acid,<sup>4</sup> as well as salicylic acid, is more volatile in steam than the above compounds. Serious interference by these acids is not likely to be experienced, however, since both are but slowly oxidized by ceric sulfate in the presence of palladium, and both may be largely removed from experimental samples by taking advantage of their low solubility in acid solution and by redistillation.

$\beta$ -Hydroxypropionic and  $\beta$ -hydroxybutyric acids, prepared in the laboratory from the corresponding halogen derivatives, were tested both by direct oxidation, and also by distillation followed by oxidation of the distillate. Neither of these hydroxy acids is oxidized at an appreciable rate in the absence of the catalyst. Both are volatile in steam, and both are oxidized by ceric sulfate in the presence of the catalyst, the rate of oxidation being about one-tenth that of formic acid. Only the latter,  $\beta$ -hydroxybutyric acid, is likely to occur in biological samples, and an indication of the error which it may cause in recoveries of formic acid is given by the data in Table III.

<sup>3</sup> High recoveries of formic acid from samples containing glucose were obtained only when the sample taken for distillation was acidified with a gross excess of sulfuric or phosphoric acid.

<sup>4</sup> Fumaric acid, another  $\alpha,\beta$ -unsaturated compound, is also oxidized slowly in the presence, but not in the absence, of palladium.

Both lactic and pyruvic acids are volatile in steam, about 25 per cent of the former and 90 to 100 per cent of the latter being obtained in the distillate under the experimental conditions employed here. These compounds were studied further by taking for oxidation samples containing a constant amount (10 micromoles) of formic acid plus graded amounts of

TABLE III  
*Recovery of Formic Acid in Steam Distillates*

Compounds distilled		Formic acid found	
<i>micromoles formic acid</i>		<i>micromoles</i>	<i>per cent</i>
200		203	101.5
300		296	98.7
	500 micromoles cinnamic acid	100	
150	+ 5 ml saturated solution cinnamic acid, redistilled	151	100.7
	500 micromoles ethylene glycol + 500 micromoles propylene glycol	4	
200	+ 200 micromoles glucose	198	99.0
	500 micromoles glycerol	5	
300	+ 300 micromoles glycerol	296	98.7
	500 micromoles glycolic acid	71	
	500 " " " redistilled	15	
100	+ 100 micromoles glycolic acid, redistilled	100	100.0
	200 micromoles $\beta$ -hydroxybutyric acid	70	
	200 " " " redistilled	38	
200	+ 100 micromoles $\beta$ -hydroxybutyric acid, redistilled	215	107.5
	200 micromoles $\beta$ -hydroxypropionic acid	78	
	200 " " " redistilled	19	
200	+ 200 micromoles $\beta$ -hydroxypropionic acid, redistilled	217	108.5
200	+ 200 micromoles lactic acid	204	102.0
	500 micromoles levulinic acid	15	
200	+ 200 micromoles levulinic acid	201	100.5
120	+ 120 " " " redistilled	122	101.7

\* Corrected by the factor 1.04

the other acids. Satisfactory recoveries of formic acid ( $100 \pm 2$  per cent) were obtained from samples containing up to 5 micromoles of lactic acid or 10 micromoles of pyruvic acid, *i.e.*, a total of 20 microequivalents of extraneous compounds. With larger amounts of lactic or pyruvic acid, more  $\text{CO}_2$  was found than that required by theory.

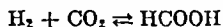
*Determination of Formic Acid in Bacterial Cultures and Suspensions*  
—*Shigella sonnei* was grown at  $35^\circ$  in 1 per cent glucose broth, and in 1 per



2 N NaOH, and centrifuged. Aliquots of each supernatant fluid were then assayed for lactic, pyruvic, and formic acids. Lactic acid was determined colorimetrically according to the method of Barker and Summerson (19). Lactic acid determinations on pure samples of pyruvic acid indicated that the amount of the latter acid present in the experimental samples would not cause a serious error in recoveries of lactic acid. Lactic acid plus pyruvic acid and formic acid were determined on the same sample by manometric oxidation with ceric sulfate. Double side arm vessels were used for this, the acidified sample being placed in one side arm, catalyst in the second, and ceric sulfate in the main well. After equilibration, the sample was tipped into the ceric sulfate and readings were taken to determine the amount of  $\text{CO}_2$  produced from lactic and pyruvic acids. The catalyst was then tipped in and readings were taken for the amount of  $\text{CO}_2$  produced from formic acid. Alcohol and acetic acid were not determined, both because of the small amounts of these compounds calculated to be present and also because the initial purpose of this experiment was not to obtain a complete fermentation balance for pyruvic acid. Data for formic and pyruvic acids were not obtained in Experiment 3 (Table V), since the phosphate present here would have interfered by precipitating ceric sulfate from solution.

A final experiment, intended to show the application of this procedure to the microdetermination of formic acid, was patterned after Woods' (7) critical studies on the degradation and synthesis of formic acid by *Escherichia coli*. Woods employed the gravimetric procedure for determination of formic acid and could not, therefore, establish a fermentation balance for each Warburg vessel used in his work.

The cells from a 15 hour culture of *Escherichia coli* in 0.5 per cent formate broth were harvested by centrifugation, washed once in distilled water, resuspended, aerated 4 hours, again centrifuged, and finally resuspended to a suitable volume in distilled water. Aeration of the suspension served both to reduce subsequent endogenous fermentation and also to inactivate the enzymes involved in the exchange reaction,



until zero time in the Warburg experiments. This inactivation is reversible (20), reactivation was effected here, if desired, by exposing the cells to sodium formate (see the protocol, Experiments 2 to 4, Table VI).

The manometer readings were followed in this experiment until the exchange reaction approached equilibrium, after which the acid was tipped in to obtain final bound  $\text{CO}_2$ . The contents of each vessel were then taken for determination of formic acid as in the work with *Shigella dysenteriae* above. In calculating the results obtained from this experiment, corrections for the controls were made by assuming the endogenous fermentation to be entirely

glycolytic in nature The data (Experiments 1 and 5, Table VI) were, within the limits of experimental error, in agreement with this assumption. The corrected data for hydrogen, carbon dioxide, and formic acid exchange are in agreement with those obtained by Woods, and adequately illustrate that the manometric procedure for determination of this acid is applicable to such studies.

TABLE VI  
*Fermentation and Synthesis of Formic Acid by Escherichia coli*

	Experiment 1	Experiment 2*		Experiment 3		Experiment 4	Experiment 5
	95 per cent N <sub>2</sub> -5 per cent CO <sub>2</sub>	95 per cent H <sub>2</sub> -5 per cent CO <sub>2</sub>				N <sub>2</sub>	
	ml	ml		ml		ml	ml
Additions to vessels							
0.05 M NaHCO <sub>3</sub>	0.5	0.9		0.1			
0.2 " phosphate buffer, pH 7						0.5	0.5
0.05 " Na formate		0.1		0.9		0.2	
H <sub>2</sub> O	1.3	0.8		0.8		1.1	1.3
Bacterial suspension (side arm)	0.2	0.2		0.2		0.2	0.2
Ag <sub>2</sub> SO <sub>4</sub> -H <sub>2</sub> SO <sub>4</sub> (second " " )	0.1	0.1		0.1			
25% KOH (inset)						0.1	0.1
	microliters	micro-liters	micro-liters	micro-liters	micro-liters	micro-liters	micro-liters
Gas exchange							
"Metabolic" CO <sub>2</sub>	+29						
" " H <sub>2</sub>			-376		+385	+222	+6
Bound CO <sub>2</sub> , final	851	1174		559			
" " initial	886	1582		186			
" " difference	-35	-408	-408	+373	+373		
Formate							
Added	0	112		1008		224	
Found	22	489		627			
Difference		+377	+377	-381	-381		

\* All data given for Experiments 2 and 3 are corrected for the endogenous fermentation of the control (Experiment 1), the data for Experiment 4 are corrected for the respective endogenous fermentation (Experiment 5).

#### DISCUSSION

The experimental conditions adopted as most satisfactory for the determination of formic acid were 1 ml of 10 per cent ammonium tetrasulfatocerate ( $Ce^{4+} Ce^{3+} = 4$ ) in *N* H<sub>2</sub>SO<sub>4</sub>, 1 ml of sample in *N* H<sub>2</sub>SO<sub>4</sub>, 0.2 ml of 10 per cent palladinized asbestos, and a working temperature of 35°. Other salts of cerium were used during the initial stages of this study, but

these appeared to be less satisfactory. Anhydrous ceric sulfate, for example, gave quite large blanks in the control vessel, and this could not be attributed entirely to an increased formation of molecular oxygen, since the blank was reduced by placing alkali in the inset of the Warburg vessel. Ammonium hexanitratocerate was also tested and found to be a less satisfactory oxidant than the sulfate.

The ammonium tetrasulfatocerate was used both as a 10 per cent and also as a 6.4 per cent (approximately 0.1 N) solution in  $N$   $H_2SO_4$ . The electrode potential of the former solution was better poised and, therefore, permitted more consistent recoveries of formic acid. Such solutions, after partial reduction with hydrogen peroxide, were found by titration with ferrous sulfate to be 0.13 to 0.15 N in respect to ceric ion.

The concentration of sulfuric acid in the vessels, after the oxidant, sample, and catalyst were mixed, should be 0.5 to 1.0 N for best results. Higher concentrations, 2 and 5 N  $H_2SO_4$ , lowered the blank, but at the same time both decreased the rate of oxidation of formic acid and also inactivated the catalyst. Lower concentrations, 0.5 to 0.1 N  $H_2SO_4$ , appeared to increase the rate of oxidation of water more than that of formic acid.

Aluminum oxide and platinum were tested as catalysts for this reaction. The former was entirely inactive. Both platinized asbestos and platinized barium sulfate catalyzed the oxidation of formic acid but the rate was too slow to be practical. Palladinized barium sulfate, prepared according to Schmidt (21), was considerably more active than platinum but somewhat less active than a comparable amount of palladinized asbestos. The latter catalyst was tested in amounts ranging from 0.05 to 0.5 ml of a 10 per cent suspension per vessel. More than 0.15 to 0.20 ml did not significantly shorten the time required for complete oxidation of the formic acid present, but rather served only to increase the amount of water oxidized. The catalyst, when suspended in 0.10 to 0.01 N  $H_2SO_4$ , showed no decrease in activity after 7 months standing. Suspensions in  $N$   $H_2SO_4$  became inactive in a few weeks, presumably due both to loss of palladium by oxidation and also to coating of the metal with a film of palladium sulfide (22). The catalyst taken for determination of formic acid may be recovered and used three or four times without serious loss in activity. We accomplished this by pooling the contents of the vessels, upon completion of a series of determinations, centrifuging, washing once with 0.1 N  $H_2SO_4$ , and finally bringing into 10 per cent suspension by adding 4 volumes of 0.1 N  $H_2SO_4$  to the wet residue.<sup>5</sup>

Many biological compounds are oxidized to formic acid by ceric sulfate, and hence samples cannot, as a rule, be taken directly for determination. The type reactions for this group of compounds have been adequately defined by Smith (10). Most of these, however, may be removed completely

<sup>5</sup> 1 gm. of palladinized asbestos has a wet volume of 2 ml.

by steam distillation of the sample. Other compounds which may interfere, if present in samples taken directly for determination, are the  $\alpha,\beta$ -unsaturated carboxylic acids, compounds which have been shown (23) to be activated by catalytic amounts of palladium. More than a trace of these (e.g., fumaric, maleic, and cinnamic) is seldom encountered in bacterial cultures and this can be removed fairly readily by steam distillation.

## SUMMARY

A procedure was described for the manometric determination of formic acid by oxidation with ceric sulfate in the presence of catalytic amounts of palladium. The error, with 0.5 mg. of formic acid, was less than  $\pm 3$  per cent. Compounds which might interfere in this determination were investigated and methods were given for determining formic acid in samples containing these compounds. The procedure was shown to give satisfactory recoveries of formic acid in bacterial cultures and suspensions.

We are indebted to Dr. C. E. Clifton for the acetone, 2,3-butylene glycol, and diacetyl used in this work.

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# EFFECT OF A CONCENTRATE OF POTENTIAL STREPTOCOCCUS LACTIS R-STIMULATING FACTOR ON GROWTH AND WHITE BLOOD CELL COUNTS OF SUCCINYLSULFATHIAZOLE-FED RATS\*

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In a recent communication (1) from this laboratory it was shown that the distribution of vitamin M is different from that of folic acid. However, materials which contained very little folic acid but were good sources of vitamin M were found to give an increase in folic acid (as measured by *Streptococcus lactis* R assay) when incubated with fresh rat liver (2) or with an extract prepared from rat liver (3). There is other evidence indicating a relationship between *Streptococcus lactis* R-stimulating factor and vitamin M (4, 5). It was suggested by Totter and Day (6) that the leucopenia of rats fed succinylsulfathiazole might have an etiology similar to that of nutritional cytopenia (vitamin M deficiency) in the monkey. The suggestion was based on our observations that xanthopterin fed to the nutritionally cytopenic monkey is followed by white blood cell and reticulocyte responses (1) and that occasionally we have obtained similar although short lived responses in succinylsulfathiazole-fed rats (6). Evidence has been presented showing that xanthopterin is closely related to folic acid, a *Streptococcus lactis* R-stimulating factor (7).

Experiments have now been made in which liver extract and yeast extract were used as sources of the factor antagonistic to the leucopenic effect of succinylsulfathiazole in rats. The results are in accord with the view that the rat antisuccinylsulfathiazole factor is the same or very similar to vitamin M. The factor is not measured by direct microbiological assay but apparently may be determined by enzymatic treatment followed by microbiological assay as suggested by Mims, Totter, and Day (3).

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## EXPERIMENTAL

Preformed *Streptococcus lactis* R-stimulating substance was determined by the assay method for folic acid of Mitchell and Snell (8) Potential

TABLE I

Growth and White Blood Cell Data on Albino Rats Receiving Purified Diet Containing 1 Per Cent Succinylsulfathiazole and Various Supplements for 8 Week Period

Group No *	Daily supplement	Streptococcus lactis R stimulating factor		Average gain in weight	Experi- mental day	White blood cells	Granu- locytes
		Pre formed	Potential				
		$\gamma$ †	$\gamma$ †	gm		per c mm	per c mm
I (4)	None			34 0	39	5,088	225
					46	5,170†	236†
					57	5,850	188
II (5)	3 mg liver extract	0 076	0 126	57 5	39	5,362	208
					46	4,975§	393§
					57	5,406§	423§
III (5)	12 " " "	0 304	0 504	53 2	39	4,820	87
					46	3,520§	241§
					57	4,631§	333§
IV (5)	20 " yeast "	0 02	1 6	81 2	39	10,393	1197
					46	6,255	980
					57	7,762	370
V (5)	40 " " "	0 04	3 2	100 0	39	11,440	1814
					46	9,055	1636
					57	7,762	1592
VI (5)	5 $\gamma$ biotin			24 8	41	4,285	231
					52	4,027†	163†
VII (5)	5 " " and 7 2 mg yeast concen- trate	0 037	3 7	109 8	41	6,960	689
					52	9,210	1146
VIII (5)	5 $\gamma$ biotin and 14 4 mg yeast concen- trate	0 074	7 4	138 6	41	8,395	1222
					52	9,865	901

\* The numbers in parentheses indicate the total number of animals in the group

† The values are in terms of folic acid of potency 40,000

‡ Average for the three surviving experimental animals

§ Average for the four surviving experimental animals

*Streptococcus lactis* R-stimulating factor was estimated by the method of Mims, Totter, and Day (3)

Concentrates of potential *Streptococcus lactis* R-stimulating factor were prepared from yeast extract<sup>1</sup> by adsorbing with Super Filtrol or norit at pH 3 to 4 and eluting with 2 per cent ammonium hydroxide or with a pyri-

<sup>1</sup> Bacto-yeast extract, Difco Laboratories, Inc., Detroit

dine-ethanol-water mixture (1 2 2) The eluates were evaporated to dryness, dissolved in water, and precipitated by adding alcohol The active material was usually found in the 60 per cent alcohol-soluble, 80 per cent alcohol-insoluble fraction One of these preparations was tested by feeding to succinylsulfathiazole-fed rats A second adsorption on Super Filtrol and elution with ammonia afforded much more active material but the loss was too great The most active preparations obtained contained 4 mg of material of potency 40,000 per gm of total solids This represented a 52-fold concentration of the potential factor from the yeast extract In this preparation the preformed factor was concentrated only 10-fold

For the animal assays rats from our stock colony (Sprague-Dawley strain), weighing 45 to 75 gm, were fed a ration with the following percentage composition sucrose 74, casein 18, cottonseed oil 3, salts 2 (9), cod liver oil 2, and succinylsulfathiazole 1 1 gm each of inositol and choline chloride were added to each kilo of diet The other B vitamins were fed daily to all animals in supplement dishes at the following levels thiamine chloride 50  $\gamma$ , riboflavin 34  $\gamma$ , pyridoxine 20  $\gamma$ , calcium pantothenate 100  $\gamma$ , and nicotinamide 200  $\gamma$  Vitamin K (2-methyl-4-amino-1-naphthol), 10  $\gamma$  daily, was added when it seemed advisable

The rats were divided into groups controlled as to sex and litter Group I received no supplement other than the B vitamins listed above Group II received 3 mg of liver extract,<sup>2</sup> Group III received 12 mg of liver extract, Group IV, 20 mg of yeast extract,<sup>1</sup> Group V, 40 mg of yeast extract, Group VI, 5  $\gamma$  of biotin, Group VII, 7.2 mg of yeast extract concentrate and 5  $\gamma$  of biotin, Group VIII, 14.4 mg of yeast extract concentrate and 5  $\gamma$  of biotin The supplements were given daily as a water solution in small dishes

The animals were weighed weekly or semiweekly and the weights and food consumption recorded White blood cell counts and differential counts were made on the animals on the experimental days indicated in Table I The average counts for each group are recorded in Table I together with the data for the average weight gain and the content of preformed and potential *Streptococcus lactis* R-stimulating substance in each supplement

#### DISCUSSION

It may be seen from Table I that the animals receiving the yeast extract or yeast extract concentrate, both of which are very low in preformed *Streptococcus lactis* R-stimulating factor, grew markedly better and maintained higher leucocyte and granulocyte counts than those which received liver extract containing 1.9 to 15 times more of the preformed factor

<sup>2</sup> Liver extract, Lilly, Eli Lilly and Company, Indianapolis



A comparison of the average growth of the experimental groups indicates that at the levels fed there is no correlation between the amount of preformed *Streptococcus lactis* R-stimulating factor and the weight gain. Group III, which received the largest amount of the preformed factor, showed a lower weight gain than that of any other group except the controls (Groups I and VI) which received no yeast extract or liver extract supplement. Group VIII, which received approximately the same amount of preformed *Streptococcus lactis* R-stimulating factor as Group II, showed a weight gain nearly  $2\frac{1}{2}$  times greater.

On the other hand, the weight gains of the animals correlated satisfactorily with the amount of potential *Streptococcus lactis* R-stimulating factor in the supplements when the quantity of the factor fed was between 0.5 and 7.4  $\gamma$  daily (expressed as folic acid of potency 40,000). The white blood cell and granulocyte counts of the various groups indicate that a similar conclusion may be drawn with respect to the leucocytopoietic activity of the supplements.

The experimental results obtained with the succinylsulfathiazole-fed rats confirm our earlier suggestion (2) that assays for preformed folic acid are probably not applicable to the nutrition of the rat, chick, monkey, and other higher forms. Binkley *et al.* have also noted that the direct microbiological assay does not measure the chick antianemia potency of yeast extracts (10). Daft and Sebrell (11) have shown that vitamin B<sub>12</sub> and *Lactobacillus casei* factor are highly active against the leucopenia and anemia in rats caused by succinylsulfathiazole feeding. The quantities of these substances used by Daft and Sebrell, however, were much higher than the quantities of preformed *Streptococcus lactis* R-stimulating factor used in the present study.

Since there appear to be a number of *Streptococcus lactis* R-stimulating substances with varying potencies (12-15), it is probable that an assay method with succinylsulfathiazole-treated rats would give results more useful when applied to the nutritional problems of higher animals than do the microbiological assays. The results of this study, with other evidence which has accumulated from several laboratories (2, 4, 5, 11, 13), strongly suggest that the *S. lactis* R-stimulating substances (vitamin B<sub>12</sub>, norit eluate factor, folic acid) together with the substances enzymatically convertible to such factors (potential *S. lactis* R-stimulating factor, vitamin B<sub>12</sub> conjugate), the rat antisuccinylsulfathiazole factor, and vitamin M are the same or are closely related.

#### SUMMARY

A method is described for the preparation from yeast extract of a concentrate containing the factor enzymatically convertible to *Streptococcus*

*lactis* R-stimulating substances By this method a 52-fold concentration of the potential factor, relatively free from the preformed factor, was effected The effect of such yeast concentrates and of yeast extract was compared with the effect of liver extract on the growth and white blood cell counts of succinylsulfathiazole-treated rats

There was no correlation between the content of preformed *Streptococcus lactis* R-stimulating substance of the supplements at the levels fed and the growth-promoting and leucocytopoietic effects of the same supplements

On the other hand, the growth-promoting effect of the supplements and their effect on the total white blood cell and granulocyte counts of the experimental animals are satisfactorily expressed by the amount of potential *Streptococcus lactis* R-stimulating factor present in the supplements

It is suggested that the factor antagonistic to the succinylsulfathiazole effect in rats is the same as vitamin M or is similar to it Both factors appear to consist of substances which either stimulate *Streptococcus lactis* R or which may be enzymatically converted to *Streptococcus lactis* R-stimulating substances

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# THE PATTERN OF DISTRIBUTION OF CARBONIC ANHYDRASE IN THE CEREBRUM OF MAN COMPARED WITH THAT OF CERTAIN OF THE LOWER ANIMALS

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In a previous paper (1) data were given indicating that carbonic anhydrase occurs in patterns in the central nervous system more or less peculiar to the species studied, that there is in general a tendency to increases in a rostral direction, and that the enzyme is found in the white matter and is possibly more important in relation to the nerve fiber than to the nerve cell. It was pointed out that the relative quantitative occurrence found by the author in different parts of the nervous system parallels the  $O_2$  uptake reported for similar material by others. It was postulated that carbonic anhydrase has a rôle in the carbohydrate metabolism of the central nervous system and might play a part in increasing the speed of propagation of the nerve impulse either from a position within the nerve fiber or outside it, from accessory cells or material. The possibilities of such an event were discussed from the chemical standpoint.

## EXPERIMENTAL

*Technique*—The technique previously described for determining carbonic anhydrase in tissue (2) was used in part. Part of the work was done with the use of a modification consisting of a low temperature water bath capable of control to  $\pm 0.1^\circ$  and a controlled mechanical stirrer. The diluting fluid, the emulsion, the standard, and the gas were cooled in the water bath.<sup>1</sup> A temperature of  $3.0^\circ \pm 0.1^\circ$  was maintained and the blank adjusted to 66 to 70 seconds by control of the rate of stirring and the flow of  $CO_2$ .

The material studied includes sections of brains from mentally normal subjects designated with letters and several from patients of a mental hospital for which case numbers are given. The hog brains were obtained fresh from the hospital abattoir. Of the dogs, one was killed with ether, the other with  $CO$ . One of the cats was shot, the others killed with  $CO$ .

To obtain the four different levels of the brain discussed, a gyrus was amputated at its base and white matter, which was removed from below it, was designated "subgyral white matter, D." For further study of the gyrus,

<sup>1</sup> These instruments were purchased with funds from the Supreme Council, Thirty-third Degree, Scottish Rite, Masons, and the Northern Jurisdiction, U S A

vertical slices were cut. Approximately the outer half of the cortex, *A*, was removed with a sharp scalpel and collected separately, after which the remaining gray matter, *B*, was either cut or scraped from the white. The latter is designated "intragyrus white matter" or *C*. The assumption is that it represents fibers a degree nearer to their place of origin or ending, as the case may be, than the subgyrus samples. The intragyrus white matter carries some contamination of gray matter and the lower cortical sections some contamination of white matter. All data are given after correction for enzyme due to the blood content of the tissues, which in the

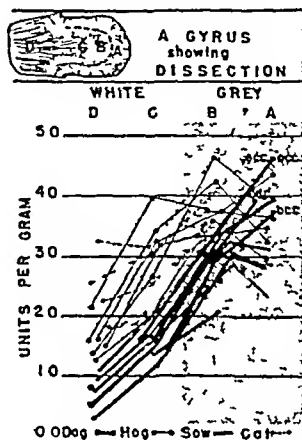


FIG 1 Distribution of carbonic anhydrase content in the cerebrum found in the cat, the dog, and the hog. *A* represents the outer half of the gray matter, *B* the inner half of the gray matter, *C* the white matter immediately below the gray matter, and *D* the white matter below the gyri. "Occ" indicates material from the caudal portion of the hemisphere.

gray matter approximated the equivalent of 1 per cent of packed corpuscles and in the white matter was less.

*Relationship of Carbonic Anhydrase Content of Cortex to That of Underlying Fibers in Brain of Dog, Hog, and Cat*—Separate determinations were made upon the gray and white matter in nine hemispheres of six hogs, six studies from the brains of two dogs, and six from the brains of four cats. In the study of the hogs material was taken from the whole hemisphere. Rostral and caudal areas of the brains of the dogs and cats were studied separately. The results are charted in Fig 1.

In practically all instances the carbonic anhydrase content of the gray matter is greater than that of the white immediately below it. The outer gray matter in dogs was also richer in the enzyme than the white matter

and in the majority of instances contained more enzyme than the inner portion of the cortex. The average content of the gray matter was 34.9 units per gm, and that of the white matter immediately below it 22.5 units per gm, giving the lower gray matter 55 per cent more carbonic anhydrase than the contiguous white matter.

*More General Relationship Found between Carbonic Anhydrase Content of White and Gray Matter in Human Brain*—In the human brain the above situation was reversed. The maximum enzyme content was not found in the cortex but in the white matter immediately below it, in the intragyral matter, and in most instances a higher content was also found in the subgyral white matter than in the cortex. The outer gray matter was always found to contain less enzyme than the inner. The average of fifteen comparisons, nine from areas of two brains from non-psychotic subjects, was for the lower gray matter 32.1 units per gm and for the contiguous white matter 40.3 units per gm, an increase of 25 per cent in favor of the white matter. In Fig. 2 is charted the enzyme content of two levels each of the gray and white matter taken from various areas of three brains. This relationship, the reverse of that found in hogs, dogs, and cats, was, with the exception to be noted, the rule in the human brain. The regions studied were the frontal, temporal, and occipital poles, the superior frontal gyrus, sections from the superior, middle, and inferior temporal gyri, approximately Brodmann's areas 7, 18, and 19, the hippocampal gyrus, and the insula. In all, 81 series of determinations in eighteen brains have given this picture.

*Motor Area*—Only when sections were taken in the vicinity of the fissure of Rolando did there appear definite exceptions to this finding in man, a maximum carbonic anhydrase content in the white matter immediately below the cortex.

The whole of the precentral gyrus was examined from four brains. From four brains material from the caudal aspect of the precentral gyrus only was tested. This included the cortex from the center of the surface of the gyrus to the bottom of the fissure of Rolando, and was obtained by splitting the gyrus with a vertical incision. Material so collected was then subdivided into the inferior and superior cortical layers and the intragyral white matter. In two instances determinations were made upon the rostral aspect alone of the precentral gyrus and once upon the rostral aspect of the postcentral gyrus. The results obtained are given in Table I.

In nine samples taken from four brains, in which the whole cross-section including both the rostral and caudal halves of the precentral gyrus was tested, the results were ambiguous. In four instances the lower strata of the cortex gave a reading equal to or greater than that of the white matter immediately below, in five the enzyme content of the intragyral white

matter was greater than that of the cortex above it. The averages of these findings are given. In the one case in which the rostral aspect of the precentral gyrus was tested by itself, approximately the same results were obtained in the lower levels of the cortex and the intragyrar white matter.

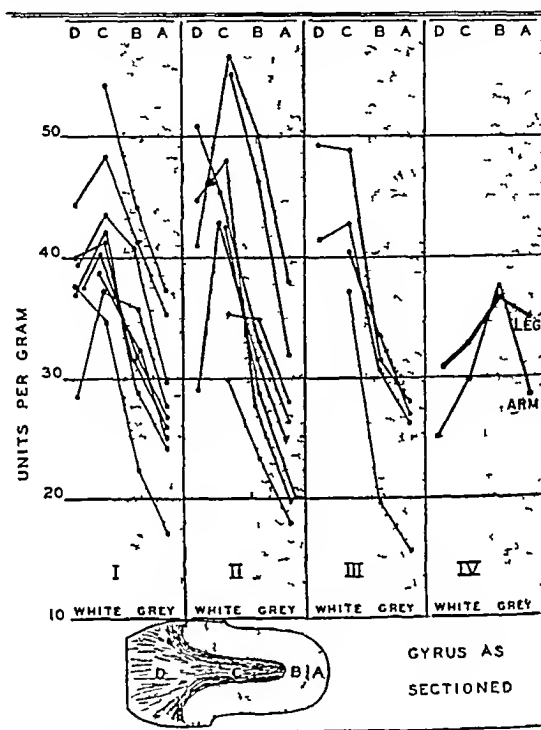


FIG 2 Carbonic anhydrase distribution in the human cerebrum. Brains I and III are from young non-psychotic subjects. Brain II is that of a patient from a mental hospital who died in good physical condition. Samples are taken from numerous areas exclusive of the motor area. In contrast IV charts the averages for the findings from the selected motor areas given in Table I. A and B represent the outer and inner strata of the cortex, C the intragyrar white matter, and D the subgyral white matter.

in both hemispheres, in contrast the caudal aspect of the gyrus of the same brain showed well defined differences in favor of the cortex.

In the four brains in which the precentral gyrus was split and the caudal aspect was tested separately, out of ten determinations nine gave a greater amount of enzyme in the cortex, eight times in the lower strata and once in the outer half. The exception in the tenth instance was in material from the left hemisphere of a man of 82, this may have been due to the

failure to locate the rolandic fissure correctly rather than to the age of the subject. It is hoped that further data will be collected pertaining to this point, but since nine out of ten trials in the more specifically located motor area give the enzyme pattern found in the animals studied, while 81 trials

TABLE I

*Relationship between Carbonic Anhydrase Content of Gray and White Matter in Rolandic Area*

Case No	Age	Hemisphere	Carbonic anhydrase units per gm			
			Gray matter		White matter	
			Outer half	Inner half	Intragyr al	Subgyral
Entire precentral gyrus						
Average of 9 specimens	37.5		26 0	33 2	35 9	28 7
Motor area (selected), leg						
A	25	Right	34 3	30 1	28 3	21 4
8121	71	Right	35 5	36 5	25 1	21 3
		Left	36 8	39 0	32 5	
8150	82	Right	30 6	35 0	32 9	
		Left	25 0	30 1	39 4	
8179	42	Right	39 9	41 0	29 5	26 0
		Left	42 5	47 9	43 0	39 6
Motor area (selected), arm						
8121	71	Right	35 3	30 2	31 1	25 0
		Left	29 5	33 0	26 5	
8179	42	Left	30 7	49 0	31 1	24 8
Sensory-motor area						
A	25	Right	29 2	35 0	28 6	23 9
Precentral gyrus, rostral aspect						
8121	71	Right	26 5	26 9	27 6	21 6
		Left	19 9	25 5	25 5	26 1

in other areas all give the reverse pattern, the finding would seem to be significant.

## DISCUSSION

The mirror of the animal pattern of distribution of carbonic anhydrase found in the human brain is difficult to understand. It would appear not



to be due to any differential deterioration of the material, since in one instance autopsy was performed on the human subject only 2 hours after death, while the typical animal pattern was still found in brains removed from animals 24 hours after death. The fact that the animal pattern is found in the motor area of the human brain would also be against such an explanation. The human pattern is probably not associated with a large brain *per se*, because the brain of the steer, which is large compared with that of the dog, still apparently shows the animal pattern, although work upon its differential enzyme content is unsatisfactory, as the total content is extremely low. The line of demarcation between the white and gray matter in the fresh brain is quite well defined and, although in the softer brain of the hog, for instance, it is somewhat more difficult to make the dissections, it is not believed that there has been any radical difference in handling that would account for the findings. Neither is it probable that the proportionately more extensive motor area found in the brains of animals accounts for this phenomenon, since the animal pattern was found in caudal quarters of the brain of the dog and cat.

It was first thought possible that this mirror of the pattern found in animals was a peculiarity of the brain of the psychotic subject and because of this possibility the motor area was studied, as it was assumed that the motor area would probably be functioning normally. The study of three brains of non-psychotic subjects, however, has revealed this same pattern. The brains of other Primates have not yet been available.

If it may be assumed that carbonic anhydrase plays a part in the metabolism involved in the propagation of the nerve stimulus, it would follow, since the nerve impulse is a surface phenomenon, that with division of a nerve fiber there would need to be a greater energy output per cubic area to maintain the same head of energy per plane area, a minimum value for which would be necessary for the continuation of the propagation. If in the animals there is a comparatively direct and narrow connection between the lower levels and the cortex, one would expect the greater amount of energy production per cubic area in that region in which there is a great subdivision of the afferent nerve as it makes contact with the cell bodies of the cortex, which, if carbonic anhydrase is a factor in this nerve metabolism, would bring the maximum carbonic anhydrase content within the cortex. If, however, the connection with the cortex were more diffuse and considerable division of the afferent nerves took place as they entered the cortex, there would be a greater tendency for the carbonic anhydrase to increase in the white matter immediately below the cortex. The very considerable excess in the carbonic anhydrase of the white matter immediately below the cortex over that of the cortex suggests, however, that some other factor than that of producing the minimum energy necessary

to carry the nerve impulse onto the greater surface caused by subdivision might be involved

#### SUMMARY

1 In the hog, the dog, and the cat a greater amount of carbonic anhydrase has been found in the cortex than in the white matter immediately below it. An average ratio of 34.9:22.5 occurred in twenty series tested.

2 In the human brain, as a rule, more carbonic anhydrase was found in the white matter immediately below the cortex than in the cortex. This was the relationship in 81 series from eighteen brains.

3 Exception to this excess was found in the motor area of the human brain where the relationship was that found in the animal brains.

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# ON THE DISTRIBUTION OF CARBONIC ANHYDRASE IN THE CEREBRUM

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(Received for publication, August 2, 1944)

In previous papers (1, 2) data were presented indicating that carbonic anhydrase tends to have a pattern of quantitative distribution in the central nervous system somewhat peculiar to the species studied but, in general, shows an increase rostrally. In the dog, the cat, and the hog, a steep gradient of increase was found from the brain stem to the pallium. In man this was apparently not the case, the average content of the pallium being equal to, or less than, that for the rostral end of the pons. Man also differed from the above animals in that, with the exception of the motor area, the maximum carbonic anhydrase content of the pallium was found immediately below the cortex, while in the animals studied it was found within the cerebral cortex. It was postulated that the carbonic anhydrase in the tissues of the central nervous system might play a part in determining the speed with which energy was made available for nerve impulse conduction, and might therefore determine dominance of an area and the degrees of radiation of an impulse.

Further study of the human cerebral hemispheres follows

## EXPERIMENTAL

This work was done principally upon human brains, but data from the cerebrums of dogs and guinea pigs are included. The nine human brains specifically cited were in six instances normal grossly, and revealed no microscopic abnormality in sections studied. The specimens, Cases A, B, and C, came from general hospitals, the remainder was from patients dying in a mental hospital. Three had suffered from functional psychoses and are assumed to have been anatomically normal, three died with the diagnosis of senile psychosis, their brains, however, showing only slight senile changes. The approach to physiologic normality of these brains is, however, an unknown quantity. Greater weight has been given to the apparently more normal material.

All deaths took place in hospitals equipped with adequate morgues. The respective physical conditions before death, the ages of the subjects, and the time between death and autopsy were, briefly, as follows. In Case A, age 25 years, and B, age 8, in which a hemisphere and one frontal lobe

respectively were received, death resulted from accidental causes. Autopsy was performed the day of the death, which in each instance resulted 2 days after the accident. In Case A, a white male, the cause of death was carbon tetrachloride poisoning and the patient was conscious until shortly before death. Analysis showed carbon tetrachloride to be in the liver. Death in Case B was due to a concussion. The third brain, Case C, from a Negress, age 58, was obtained 2 days after death. The patient had been in a deep coma for 16 days and had died of bronchopneumonia. The kidney showed arteriosclerosis, the eye grounds could not be visualized. Three of the remaining brains were from subjects who died suddenly, two from coronary occlusion and one from cardiac decompensation. In Case 8026, age 82, autopsy was performed  $1\frac{1}{2}$  hours after death, in Case 8121, age 71, 5 hours after death, in Case 8076, age 35, 41 hours after death. Case 7991, age 82, died 2 weeks after an attack of bronchopneumonia, and autopsy was performed 8 hours after death, in Case 8179, age 42, death was due to tuberculosis, with autopsy 8 hours after death. In Case 8150, age 82, death resulted from pyelonephritis and acute bronchopneumonia, with autopsy 17 hours after death.

Of the animals, Dogs 1 through 4 were from the city pound and were killed with CO. To this method of sacrifice there seems no *a priori* objection, since carbonic anhydrase is not an oxygen-combining enzyme and does not contain iron or copper. Dog 5 was killed with ether. It was an old, but well nourished and intelligent animal. The guinea pigs were either etherized or drowned.

The technique previously described (3) was used. In the study of the human cerebrum no attempt was made to establish the validity of the named areas by microscopic examination. When the Brodmann's areas are used, the position of the material selected for enzyme titration was compared with a chart of the brain by Brodmann and a presumptive designation was given. Other designations, such as frontal, temporal, and occipital pole, the temporal gyri, names of regions readily recognized, were also used. In obtaining material for study of dog brains, each hemisphere was cut into four sections, a back, front, top, and side section, to which the designations of frontal, occipital, parietal, and temporal sections have been given. The brains of the guinea pigs were bisected into a rostral and caudal part. The enzyme content is given after subtraction of that fraction due to blood in the tissue.

*Probability of Normal Pattern of Enzyme Distribution with Reference to Topography*—In Fig 1 are plotted data from the carbonic anhydrase determinations on mixed portions from two brains, both from moderately youthful subjects whose deaths were not preceded by a long illness, the mixed portion, which contains both white and gray matter, as previously

explained (4), is part of a gyrus amputated at its base. Exclusive of the motor and sensory-motor areas, six of the same locations were studied in each brain, the frontal, temporal, and occipital poles, area 18, the parietal area 7, and a lateral temporal area. In the six areas there is shown a similarity in the pattern of the quantitative carbonic anhydrase content. The temporal pole contains the least amount of enzyme, the frontal pole and the lateral temporal areas are about equal in content, the parietal area has more than the frontal, and the areas in the occipital lobe have the highest content, with area 17 having a higher content than area 18.

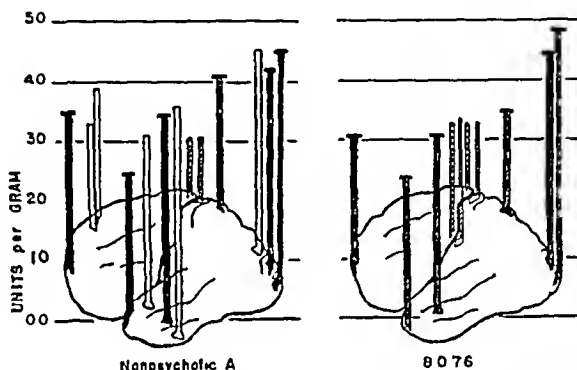


FIG 1 Illustrating the comparable patterns of carbonic anhydrase content by sections of gyri taken from the brain of a non-psychotic subject, age 25, and of a patient dying in a mental hospital, age 35. The bases of the columns rest upon the approximate areas from which the samples were chosen. The columns reach to the ordinates representing the enzyme contents of these samples. Hatched columns indicate motor and sensory-motor areas, the cross-hatched columns motor areas, otherwise determinations common to the two brains are represented by solid black columns.

In Fig 2 is plotted a summary of all data obtained to date with the exception of those on four brains which seem of doubtful quality for inclusion in the series. Data from nine brains are included in Fig 2, in four instances material from both the right and left hemispheres was available. In order to bring the data from the different brains within the same range, a factor was found which, when applied to the enzyme content of a frontal pole, gave 25 units. This factor was then applied to the rest of the data from that brain. Therefore in charting, 25 units per gm are taken as the content for all frontal poles. Several of the brains were from old and, mentally, extremely abnormal subjects, nevertheless the pattern for the average conforms to that seen in Fig 1.

In Fig 3 are plotted the findings on material taken from the four different

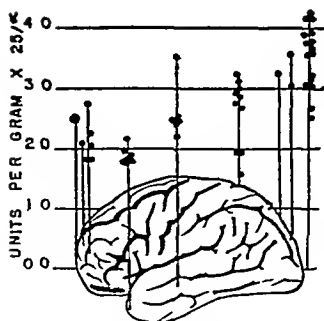


FIG 2 Carbonic anhydrase content in units per gm of mixed samples from nine brains (thirteen hemispheres) multiplied by the factor,  $25/\alpha$ , where  $\alpha$  is the carbonic anhydrase content of the respective frontal pole. Small circles indicate the individual determinations multiplied by the factor and are placed over the approximate areas from which the samples were taken.

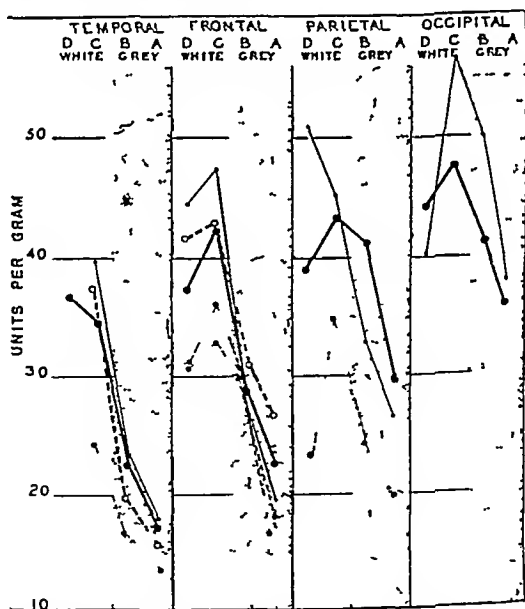


FIG 3 Comparison of carbonic anhydrase findings in the temporal, frontal, and occipital poles and in area 7. Specimens were taken from the outer half of the cortex, A, the inner half of the cortex, B, white matter within the gyrus and immediately below the cortex, C, the white matter below the gyrus, D. Heavy solid lines, Case A, heavy broken lines, Case B, fine solid lines, Case 8076, fine broken lines, Case C.

strata of the pallidum previously defined (2), the outer half of the gray matter, the inner half, the intragyral white matter, and the subgyral white matter. These samples were taken from the frontal, the temporal, and the occipital poles and the parietal area 7. Again the temporal pole has the lowest enzyme content, the occipital pole the highest, and the parietal area is intermediate between the frontal and the occipital poles. In the brain of Case A, which offers the most normal material in the series, in which all four areas were studied, a gradient is also found in the difference between the greatest amount and the least amount of carbonic anhydrase found in the frontal, parietal, and occipital areas respectively. In the frontal pole this difference is 18 units per gm, in the parietal 14 units per gm, and the occipital area 13 units per gm. This apparently indicates a general tendency since, in four other brains, three with material available from both hemispheres in which these strata were dissected out and tested separately, the difference was in all instances greater in the frontal than in the occipital pole. The average difference between the carbonic anhydrase content of the outer half of the cortex and that of the white matter immediately below the cortex in the frontal pole was 12.5 units per gm, that for the occipital pole was only 4.8 units per gm. These differences of distribution of the enzyme content in the occipital and frontal poles are seen to a greater extent as differences in content of the gray matter but they are also seen to a less extent in the white matter.

*Findings in Right Versus Left Hemispheres*—With the exception of the brain of Case C from a woman in a coma for 16 days before death, material from the right and left hemispheres of brains of subjects without known mental sickness has not been available to date. In the mixed sample, this brain showed a negligible difference between material taken from the right and left frontal poles. In the brain from Case 7991, that of an 82 year-old female, there was also practically no difference. In four other brains, in two of which two areas of each were studied, the average difference for the frontal region was 3.3 units per gm, while for the whole series the average was 2.6 units per gm in favor of the left hemisphere. Four brains in the series in which the outer cortex was determined separately showed some preponderance of enzyme in the left side in one instance with an average of 2.05 units per gm, but the subgyral white matter (pure white matter further away from the cortex than the intragyral white matter) gave in one instance 4.8 units per gm in favor of the left side, but in the other three the finding was  $\pm 0.7$  unit per gm.

In the occipital lobe the findings were quite different, the differences between the right and left sides were greater, averaging 9.9 units per gm and the preponderances were in either hemisphere but more often in the right than in the left hemisphere. The results for both the frontal and occipital lobes are given in Table I.



TABLE II

*Carbonic Anhydrase Distribution in Nine Hemispheres from Five Dogs*

Dog No	Hemisphere	Carbonic anhydrase units per gm			
		Frontal	Temporal	Parietal	Occipital
1	Right	24 6	19 1	21 7	24 6
	Left	25 5	22 9		
2	Right	22 1	19 1	27 4	25 5
	Left	21 7	12 6	16 0	22 4
3	Right	16 9	10 6	13 5	13 9
	Left	12 1	11 5	13 9	9 8
4	Right	30 0	25 6	24 0	26 3
	Left	24 4	21 0	26 6	23 8
5		39 5	34 9	26 9	30 7
Average		24 1	19 7	21 2	22 1

7 determinations in human brains

	Frontal pole	Temporal pole	Area 7	Area 17
Average	29 5	21 7	32 5	35 2

TABLE III

*Carbonic Anhydrase Distribution between Rostral and Caudal Halves of Brains of Twelve Guinea Pigs*

Animal No	Carbonic anhydrase units per gm	
	Rostral half	Caudal half
1	23 3	34 8
2	17 4	31 7
3	23 7	57 3
4	23 8	33 0
5	23 6	27 8
6	20 0	22 8
7	28 1	29 8
8	26 6	26 0
9	30 9	43 0
10	25 2	35 1
11	31 1	35 8
12	28 8	28 9
Average	25 6	33 8

Twelve guinea pigs were also tested. The carbonic anhydrase was determined separately in the anterior and posterior halves of the brain.

The results are given in Table III. The average carbonic anhydrase content of the occipital half of the brains was approximately 30 per cent more than that of the frontal half.

#### DISCUSSION

These data upon the quantitative occurrence of carbonic anhydrase in the pallium in the areas studied make a pattern sufficiently definite to suggest that there may be a typical pattern of quantitative distribution of the enzyme in man.

The sections of this pattern to which a more considerable part of the data have contributed imply a low enzyme content in the temporal pole, a high content in the occipital pole, the frontal pole, and the lateral temporal area approximately equal to each other and lower in content than the occipital pole, the parietal area intermediate between the frontal and occipital poles. A difference between the carbonic anhydrase content of the right and left sides of the brain giving a slight preponderance for the left side in the frontal area and a greater preponderance, but in favor of either side, for the occipital area, also may be characteristic.

The differences between the right and left hemispheres in the frontal region were near to the limit of error of the technique. They were, however, in this series, consistently in favor of the left side, except in two instances in which there was no difference. The finding, therefore, aligns with dominance of the left hemisphere. The greater preponderance found in two instances in the right occipital region with left preponderance in the frontal pole does not fit into a conception of lateral dominance. The results, however, are in harmony with studies of Raney (6) on the electroencephalogram of identical twins in which he finds no relationship between the bilateral differences found in the central area and those found in the occipital.

The finding of a comparatively lower enzyme content in the occipital area of the dog, whose proportionate mental components in the visual modality must be considerably less significant than those of man, is in support of a parallelism between mental function and carbonic anhydrase content. The finding of a larger amount of carbonic anhydrase in the occipital than in the frontal half of the guinea pig brain is more difficult to evaluate. The guinea pig is accredited (7) to be too stupid an animal to train, and there seems to be no information available as to the importance of vision in its mental makeup. The data illustrate the tendency already noted (1) of patterns of distribution of the enzyme varying with species.

The pattern of intensity of activity seen in the electroencephalogram shows a good correlation with the patterns found for carbonic anhydrase.

The relationship of the heights of  $\alpha$ -waves obtained when monopolar leads are placed upon frontal, parietal, and occipital areas (8) bears a striking resemblance quantitatively to the relative carbonic anhydrase content of the same regions as illustrated in Figs 1, 2, and 3. Also, bipolar leads connected across two symmetrical frontal and occipital areas give quantitatively similar ratios to the differences between carbonic anhydrase contents of the right and left hemispheres in samples taken from the frontal and occipital poles. Data charted in Fig 4, indicating the differences of amount of carbonic anhydrase content in the right and left hemispheres found in samples taken at various distances from the frontal pole, show a marked resemblance in their quantitative relationship to variations in the  $\alpha$ -wave obtained by Rubin (9) with horizontally placed bipolar leads also taken at points varying in distance from the frontal area. With reference to the distribution of carbonic anhydrase in the brains of dogs may be cited the findings of Bartley. Bartley (10), in studying the quantitative distribution of action currents in the brains of dogs, finds a pattern characterized chiefly by "a concentration of energy extending from the anterior to the posterior pole through the auditory area with a maximum at each of the poles." The carbonic anhydrase distribution found by me in dogs is in good agreement with this pattern. If, as I have postulated (1), carbonic anhydrase in the central nervous system has to do with a capacity for speed of delivery of energy for propagation of nerve impulse, one would expect such a correlation with the energy differences shown in the electroencephalogram.

#### SUMMARY

Data indicating a pattern of distribution of carbonic anhydrase in the human pallium are given.

The functional significance of this pattern is discussed in relation to comparative findings in the cerebrum of the dog, comparative findings in the right and left hemispheres, and in relation to the normal electroencephalogram.

I am indebted to Dr Karl H. Langenstrass, Lieutenant-Colonel Carl Lind, and the Department of Neurology, School of Medicine, George Washington University, for brain specimens.

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## URINARY EXCRETION PRODUCTS OF SULFAQUINOXALINE

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Following oral administration, the sulfonamides pass, in part, from the intestinal tract to the portal circulation and reach the liver. Here, they undergo a series of metabolic reactions which determine to a large degree whether or not the products of these metabolic reactions will form uroliths. If a particular metabolic product is relatively water-soluble (*e g* hydroxy-sulfapyridine glucuronide (1, 2)), it will not form kidney stones, but if the product (*e g*  $N_4$ -acetylsulfapyridine (3-5)) is comparatively insoluble, it may form urinary concretions. Knowledge of these detoxication products is, therefore, of importance in the etiology of sulfonamide urolithiasis.

Previous work indicates that the sulfonamides are excreted in a number of different forms. By direct isolation of detoxication products from urine, it has been shown that a given sulfonamide, at the dosage levels studied, is excreted unchanged (6), as an  $N_4$ -acetyl derivative (3, 4), as a mono-hydroxyl derivative (1), and as a water-soluble glucuronide or ethereal sulfate of the hydroxyl derivative (1, 7). By means of a partition technique (8), these excretion products are divisible into a water-soluble group and a relatively water-insoluble group. The latter includes those products which may precipitate within the urinary tract.

Recently, a new sulfonamide, sulfaquinoxaline, was introduced (9). This drug has been the subject of pharmacological investigation, and in the course of this evaluation of sulfaquinoxaline its urinary elimination was studied (10)<sup>1</sup>. Continuing this study, we have examined the urinary excretion products and have isolated and identified three members of the relatively water-insoluble group. These were shown to be unchanged sulfaquinoxaline (I),  $N_4$ -acetylsulfaquinoxaline (II), and 2-sulfamyl-3-hydroxyquinoxaline (III). In accordance with the accompanying equation, the structure of (III) was established by the isolation of 2,3-dihydroxyquinoxaline (IV). A fourth member of this group was partially characterized, and the presence of water-soluble excretion products was noted.

<sup>1</sup> Silber, R., and Clark, I., unpublished.

$C_8H_8O_2N_2$	Calculated	C 59.30, H 3.73, N 17.29
	Found	" 59.59, " 4.05, " 17.46

The ultraviolet absorption data obtained with a solution of the hydrolysis product in 0.1 N sodium hydroxide are shown in Fig. 1 (Curve 2). Included for comparative purposes are the data obtained with the synthetic sample of 2,3-dihydroxyquinoxaline (Curve 1).

*Conjugated, Acidic Excretion Product*—Following precipitation of the hydroxysulfaquinoxaline from the *alkali-soluble fraction*, the filtrate was found to contain a material which diazotized only after hydrolysis. In order to study this product further, quantitative distribution experiments

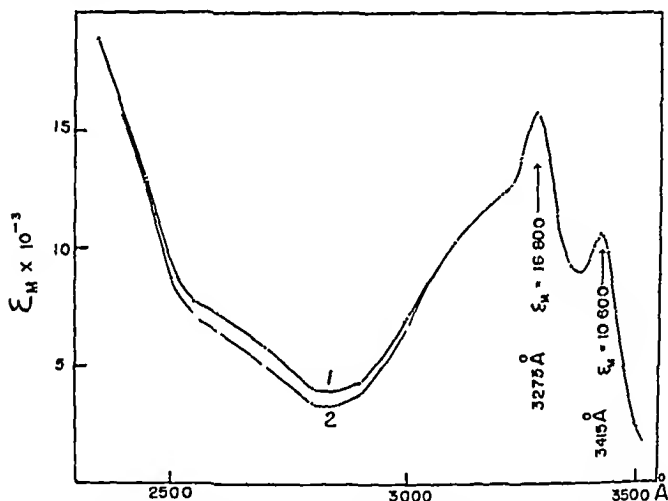


Fig. 1 The ultraviolet absorption of 2,3-dihydroxyquinoxaline in 0.1 N sodium hydroxide. Curve 1, synthetic sample, Curve 2, product isolated from the hydrolysis of 2-sulfamyl-3-hydroxyquinoxaline.

(8) were performed. Equivalent solutions of sulfaquinoxaline,  $N_4$ -acetyl sulfaquinoxaline, and the conjugated excretion product were prepared in 50 cc of 0.1 N sodium hydroxide. These solutions were shaken with equal volumes of butanol, and after equilibration both phases were analyzed. (11) Two successive determinations gave partition coefficients of 0.84, 0.80, 2.0, 2.1, and 0.17, 0.19, respectively. Thus, the conjugated acidic excretion product, assuming it to be a single substance, is not  $N_4$ -acetyl sulfaquinoxaline. Since it does not diazotize unless hydrolyzed, it cannot be unchanged sulfaquinoxaline or the hydroxysulfaquinoxaline. It exhibits the properties of 2-( $N_4$ -acetyl)-sulfamyl-3-hydroxyquinoxaline, but since the product was not isolated this structure remains tentative.

## DISCUSSION

The present investigation was limited primarily to the study of the relatively water-insoluble products that are excreted by way of the urinary system following oral administration of sulfaquinoxaline. Within this group, three products have been identified and a fourth product has been partially characterized. Among the species studied, the rabbit appears to be the only animal which excretes significant amounts of all four products. At the dosage levels used, rabbits produce urine which contains a small amount of unchanged sulfaquinoxaline, large amounts of acetylated sulfaquinoxaline and 2-sulfamyl-3-hydroxyquinoxaline, and significant amounts of what appears to be a conjugated hydroxyquinoxaline, in which the arylamino group is masked. Unlike the rabbit, the dog does not conjugate the arylamine group. Relatively little unchanged sulfaquinoxaline appears in dog urine, and so far as we have been able to ascertain, no significant amounts of the hydroxysulfaquinoxaline are present. The monkey<sup>3</sup> eliminates little of the unchanged or acetylated sulfaquinoxaline, but excretes it largely in the form of the hydroxysulfaquinoxaline. This product has been isolated and identified. It is the same as the product isolated from rabbit urine.

In the course of this work it became apparent that appreciable quantities of the urinary sulfonamide remained in solution after the samples were equilibrated at pH 4.5. In many cases, particularly with dog urine, the concentrations greatly exceeded the solubility of any of the known metabolites. This suggested, among other things, that water-soluble excretion products were present, and distribution experiments, carried out as previously reported (8), indicated that large amounts of such products appeared in rat, rabbit, dog, monkey, and human urine. These products were not isolated, but the isolation of 2-sulfamyl-3-hydroxyquinoxaline indicates, in analogy with sulfapyridine (1), sulfathiazole (7), etc., that the hydroxysulfaquinoxaline may be conjugated, in part, and excreted as water-soluble products of the ethereal sulfate or glucuronide type.

## SUMMARY

Following the oral administration of sulfaquinoxaline, the unchanged drug, *N*<sub>4</sub>-acetylsulfaquinoxaline, and 2-sulfamyl-3-hydroxyquinoxaline have been isolated from the urine of experimental animals. Evidence is presented which suggests that a fourth product, possibly 2-*N*<sub>4</sub>-acetyl-sulfamyl-3-hydroxyquinoxaline, occurs in rabbit urine. The relative occurrence of these products in the urine of various animal species has been

<sup>3</sup> Ten animals were used. These were given 75 mg. of sulfaquinoxaline per kilo of body weight by stomach tube each day.



discussed, and the presence of water-soluble excretion products of sulfaquinoxaline in the urine of the rat, rabbit, dog, monkey, and man has been noted

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# CYSTINE AND CYSTEINE IN THE WATER-EXTRACTABLE PROTEINS OF RAT AND RABBIT TISSUES

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(Received for publication, August 25, 1944)

Homologous proteins from different species appear to possess a nearly similar amino acid composition. This has been noted by several investigators as a property belonging not only to the following animal proteins, myosin (1), the neuroproteins (2), the whole muscle proteins (3), and the liver nucleoproteins (4), but to the plant proteins as well, *i e*, to the somatic proteins of the photosynthesizing tissues of related cryptogams (5). These interesting observations suggest that the homologous proteins of different species possess a nearly common amino acid plan. It is probable, however, that these similarities represent first approximations only and that very accurate analyses on highly purified protein fractions might reveal small but definite differences among such proteins (6).

More striking differences can be revealed by a consideration of the relative distribution of cystine and cysteine in these proteins. In each group of homologous proteins studied, cystine and cysteine have been estimated together by the usual classical procedures which make no distinction between them, and their sum has been very nearly the same from one homologous protein to the other. In the case of the liver nucleoproteins, in which an attempt has been made to estimate the relative proportions of cystine and of cysteine, it was observed that the cystine-cysteine fraction of the rabbit liver nucleoprotein could be accounted for entirely in terms of cysteine, whereas only one-sixth of this fraction in the rat liver nucleoprotein could be accounted for in terms of cysteine (4). Thus, although both homologous proteins contain the same total sulfur, the same methionine sulfur, and the same cystine-cysteine sulfur, the last mentioned quantity in the rabbit protein is apparently made up only of cysteine sulfur, in the rat protein it is apparently made up of both cystine and of cysteine sulfur in which cystine sulfur is in excess (4, 7). The cystine-cysteine sulfur of rabbit myosin can be accounted for nearly entirely as cysteine sulfur (7). It would appear that rabbit protein is distinguished by a relatively high proportion of cysteine. At equal protein concentration, the cysteine content of rabbit serum is considerably higher than that of rat serum (8).

In order to extend this kind of characterization of the homologous

proteins of different species, the water-extractable proteins of similar tissues of the rabbit and of the rat have been examined for their relative proportion of cystine and of cysteine. For further information, complete sulfur distribution studies have been made on the proteins obtained from the adult and the fetal livers of both species.

#### EXPERIMENTAL

The various tissues were obtained from freshly killed animals and studied immediately. The tissues were finely ground with sand in a glass mortar in the presence of 3 times their weight of distilled water. The homogenates were allowed to stand for 2 hours at room temperature with intermittent stirring and were then centrifuged at 1500 R.P.M. for 10 minutes. The supernatant fluid was decanted and used immediately for the analyses. Determinations of total nitrogen were made on each extract. Rats were fed on Purina dog chow and rabbits on rabbit chow, oats, and cabbage.

The proportion of cystine-cysteine in each extract was determined in suitable aliquots after hydrolysis by  $\text{HCl-HCOOH}$  and subsequent colorimetry with the Folin-Moreau reagent (9). Cysteine was estimated in aliquots of the various extracts by adding 1 gm. of purified guanidine hydrochloride to each cc. of the extract and by subsequently titrating the mixture with porphyrindin (10-12). The total cystine-cysteine was thus obtained on the hydrolyzed proteins, the cysteine on the intact denatured proteins. Both determinations were invariably made on the same extract. The proportion of cystine was then calculated by subtracting the value of cysteine from the value found for the total cystine-cysteine. The validity of the procedure for cysteine determination and the method of calculation have been discussed elsewhere (7). Hess and Sullivan have recently, by an ingenious procedure involving the different reducing capacities of cyanide and of sodium amalgam, demonstrated that the cysteine content determined after hydrolysis of proteins was very nearly the same as that revealed in the intact protein after suitable denaturation procedures (13). The proportion of methionine and of total sulfur in the liver tissues was determined respectively by the methods described by McCarthy and Sullivan (14) and by Greenstein and Jenrette (4). Determinations made in duplicate agreed within 10 per cent. Determinations made upon the same kind of tissue from different animals agreed within 20 per cent.

It is obvious that the various determinations include not only the sulfur-containing components of the proteins but that of glutathione as well. This is immaterial for two reasons, one, because the proportion of glutathione is small compared with the amount of protein, and two, because the sulfhydryl and disulfide groups of the protein are in equilibrium with the corresponding groups of reduced and oxidized glutathione. Indeed, it is

even preferable that the sulfur of both glutathione and the proteins be simultaneously estimated as yielding a more natural reflection of the physiological state of these tissue components

The averaged data are given in Tables I and II

TABLE I

*Cystine and Cysteine in Extracts of Normal Rat and Rabbit Tissues*

All data are given in terms of mg per mg of total nitrogen in the tissue extracts

Tissues	Cystine-cysteine		Cysteine†		Cystine†		Cystine Cysteine	
	Rat	Rabbit	Rat	Rabbit	Rat	Rabbit	Rat	Rabbit
Adult liver	0.41	0.43	0.10	0.25	0.31	0.18	3.1	0.7
Fetal "	0.26	0.11	0.06	0.05	0.20	0.06	3.3	1.2
Kidney	0.40	0.44	0.10	0.24	0.30	0.20	3.2	0.8
Spleen	0.38	0.28	0.09	0.16	0.29	0.12	3.2	0.8
Brain	0.36	0.36	0.11	0.24	0.25	0.12	2.3	0.5
Heart	0.38	0.40	0.11	0.20	0.27	0.20	2.4	1.0
Skeletal muscle	0.28	0.26	0.08	0.18	0.20	0.08	2.5	0.5
Lung	0.20	0.20	0.04	0.11	0.16	0.09	4.0	0.8
Pancreas	0.26	0.24	0.05	0.16	0.21	0.08	4.2	0.5
Average							3.1	0.7

\* By the Folin-Marenz method on HCl-HCOOH hydrolysates

† By porphyrindin titration of the proteins denatured in guanidine hydrochloride

‡ Calculated by difference, i.e., by subtraction of cysteine from cystine-cysteine

TABLE II

*Sulfur Distribution in Extracts of Adult and Fetal Hepatic Tissues*

All data are given in terms of mg per mg of total nitrogen in the tissue extracts

Liver tissue	Total S		Cystine cysteine S		Methionine S		Cysteine S		Cystine S		Cystine-cysteine S + methionine S	
	Rat	Rabbit	Rat	Rabbit	Rat	Rabbit	Rat	Rabbit	Rat	Rabbit	Rat	Rabbit
Adult	0.136	0.131	0.109	0.115	0.022	0.016	0.026	0.067	0.083	0.048	0.131	0.131
Fetal*	0.130	0.072	0.069	0.030	0.055	0.026	0.016	0.008	0.053	0.022	0.124	0.056

\* From rats 2 weeks and from rabbits 20 days pregnant

## DISCUSSION

The data in Table I reveal that although the total cystine-cysteine is very nearly the same in similar tissues of both species of animals, the relative amounts of cystine and of cysteine in this fraction are quite different. Rabbit protein in general is distinguished from rat protein by a

considerably higher proportion of cysteine than of cystine. This difference is exhibited not only in the adult but in the fetal tissues as well. These findings are thus in accord with the earlier studies on the liver nucleoproteins of the two species (4) and are in harmony with the observations of a high sulfhydryl content in the proteins of the rabbit (8, 15). This type of analysis could be extended to the tissue proteins of still other species and constitutes, in addition to the serological, a simple and direct method of distinguishing the tissue proteins of each species.

The sulfur distribution of adult and fetal hepatic tissues described in Table II reveals certain differences between them. Although the total cystine-cysteine content of adult livers of both species is nearly similar, the same cannot be said of fetal livers of both species. The pregnant rats and rabbits were sacrificed at nearly the same period of gestation, namely, at about two-thirds of the normal term. However, at this point, the total sulfur content, the cystine-cysteine content, and the methionine content of the rabbit were all roughly half those respectively of the rat. It is quite possible that the livers of the fetal rabbit and of the fetal rat do not develop at equal rates, and that the precaution of studying them at equivalent gestation times was taken in vain. However, as shown in Table I, the relative proportion of cystine to cysteine is the same in fetal as in adult livers of each species of animal. Even in the fetal stage, the rabbit liver can be distinguished from rat liver on this basis.

In both species, the content of both cystine and cysteine in the adult liver is higher, that of methionine lower, than those respectively of the fetal liver. In the relative proportion of all the sulfur-containing components in the adult and fetal livers, the results on both species of animals are consistent.

The application of this type of analysis to the proteins of the cancerous tissues of the animals considered in this communication is described elsewhere (16).

#### SUMMARY

The cystine-cysteine and cysteine contents in terms of mg per mg of total nitrogen have been estimated in fresh aqueous extracts of the minced tissues of rabbits and of rats. Cystine was calculated by difference. The cystine-cysteine values of similar tissues in both species were nearly the same, but the ratios of cystine to cysteine averaged 3.1 for rat and 0.7 for rabbit tissue proteins. The extracts of rabbit tissues are thus distinguished from those of the rat by a far higher relative proportion of cysteine. The ratios in fetal liver of the two species are as in adult tissues.

Studies of the sulfur distribution of adult and fetal livers of the two species of animals revealed that adult livers were higher in cystine and in

cysteine but lower in methionine than fetal livers. At equivalent gestation periods the sulfur development of the fetal liver of the rabbit was apparently not as far advanced as was the fetal liver of the rat.

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# A CONVENIENT TITRIMETRIC ULTRAMICROMETHOD FOR THE ESTIMATION OF UREA AND KJELDAHL NITROGEN\*

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(Received for publication, July 15, 1944)

The method originally described by Sobel, Yuska, and Cohen (1) for the estimation of urea and Kjeldahl nitrogen is modified for the application to ultramicro quantities. For this purpose a simple micro aeration apparatus is employed having standard side arm test-tubes made of Pyrex glass (This is illustrated schematically in Fig 1). For the inlet a 1-hole stopper equipped with a glass tubing of small diameter reaching to the bottom is used. For the outlet the side arm is connected with a rubber tubing to the inlet of the receiving tube. The ammonia is aerated over, after treatment with alkali, to the receiving tube, which contains boric acid and the indicator mixture of Ma and Zuazaga (2) in slightly different proportions. The trapped ammonia is titrated with standard acid with a capillary micro burette. The transfer of the ammonia is quantitatively completed in 20 to 30 minutes. Forty to sixty simultaneous aerations may be set up with the same pump. The material to be analyzed is digested (for Kjeldahl N) or treated with urease (for urea N) in the same tube from which the aeration is carried out and thus no transfer is required.

The method was adapted to the estimation of urea in 0.1 ml of blood serum containing 5 to 100  $\gamma$  of urea N and for the estimation of total N, albumin N, and non-protein N with 0.01 to 0.05 ml of blood serum.

## Reagents—

1. Phosphate buffer pH 7.0. 15 gm of sodium pyrophosphate, 2 gm of phosphoric acid (1.4 ml of 85 per cent  $H_3PO_4$ ), 100 ml of distilled water.

2. Urease extract (3). 10 gm of washed permutit are placed in a 1 liter Erlenmeyer flask. The permutit is washed with three 50 ml portions of 2 per cent acetic acid, then 100 ml of 2 per cent acetic acid are added. 150 ml of 0.001 N sulfuric acid and 50 gm of jack bean meal are added. The mixture is shaken for 15 minutes, 200 ml of glycerol are added, and the mixture again shaken for about 10 minutes and then put into the ice box.

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The mixture is occasionally stirred by shaking and allowed to settle overnight in the refrigerator. The supernatant fluid is then removed and centrifuged for 1 hour at 2000 R P M. The clear supernatant liquid is transferred to a bottle and kept in the refrigerator. The extract so prepared is very stable. One may also use commercially prepared urease tablets containing a buffer.

3 Indicator (2) 8 parts of 0.1 per cent bromocresol green in 95 per cent alcohol and 1 part of 0.1 per cent methyl red in 95 per cent alcohol

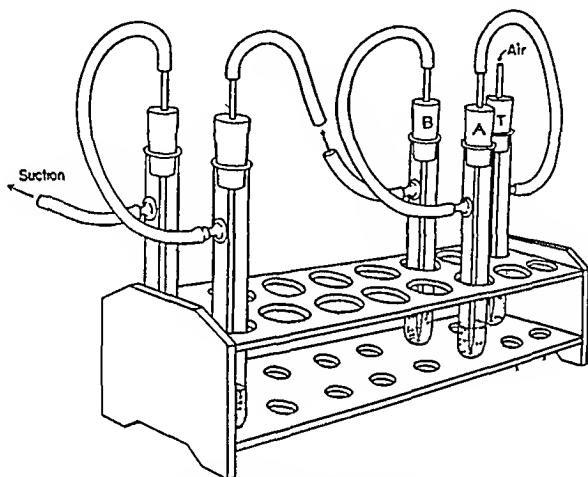


FIG 1 Schematic diagram of micro aeration apparatus.  $T$  = trap for  $\text{NH}_3$  in air (2 per cent  $\text{H}_3\text{BO}_3$ ),  $A$  = digestion tube,  $\text{NH}_4^+ \xrightarrow{\text{OH}^-} \text{NH}_3$ ,  $B$  = receiving tube, 2 per cent  $\text{H}_3\text{BO}_3$  + indicator

4 2 per cent boric acid with indicator 20 gm of boric acid are diluted to 1 liter with distilled water. 100 parts of the 2 per cent boric acid are mixed with 1 part of the indicator.

5 Urea nitrogen recovery solution 2.1434 gm of recrystallized urea are dissolved in 1 liter of distilled water. 1 ml contains 1 mg of urea nitrogen.

6 Urea nitrogen standard solution 10 ml of urea nitrogen recovery solution are diluted to 100 ml with distilled water. 1 ml contains 0.1 mg of urea nitrogen.

7 Ammonia-free caprylic alcohol saturated with thymol

8 Potassium carbonate Half saturated solution, approximately 560 gm of the anhydrous salt per liter

9 Standardized 0.0714 N sulfuric acid

10 Digestion mixture 500 ml of distilled water, 30 gm of potassium sulfate, 5 gm of copper sulfate, 480 ml of concentrated sulfuric acid, 0.5 gm of selenium in 20 ml of concentrated sulfuric acid

11 Trichloroacetic acid, analytical reagent grade, 5 per cent solution in distilled water

12 Sodium sulfate solution 23 per cent solution in distilled water This is kept in an incubator at 37°

13 Alkali solution 12 parts of distilled water, 6 parts of sodium hydroxide, and 1 part of sodium thiosulfate

14 Ether, U S P

15 Standard and recovery solution for proteins Stock solution 4.7186 gm of ammonium sulfate, which has been dried overnight at 105°, are dissolved in 500 ml of distilled water 1 ml = 10 mg Solution for use 1 ml of the stock solution is diluted to 100 ml with distilled water 1 ml = 0.1 mg

#### *Apparatus—*

1 Pyrex test-tubes with side arm (12 cm in length and 1.5 cm diameter)

2 Glass tubing, 15 to 18 cm in length, 4 mm outside diameter, with drawn out fine tip of about 1 to 1.5 mm diameter

3 1-hole size 0 rubber stoppers

4 Rubber tubing, about 33 cm in length, 8 mm outside diameter, and 3.5 mm inside diameter

5 Capillary micro burette (Rehberg, self-filling type)

6 Hot-plate

7 Electric pump or water suction

#### *Procedure for Aeration and Titration*

The digestion tube of the aeration outfit containing 10 or 100  $\gamma$  of ammonia nitrogen and 1 ml of distilled water is connected with a rubber tubing to the receiving tube, which contains 1.5 ml of the boric acid solution containing the indicator. Then 0.5 ml of the alkali solution for Kjeldahl N is added to the digestion tube, the tubes immediately stoppered, and the aeration slowly started. After 20 to 30 minutes the ammonia is completely aerated over and trapped in the boric acid, so the aeration is stopped by rapidly disconnecting the tubes from the pump. While the glass tubes, which are inserted into the receiving tubes, are being removed, they are carefully washed down with a few drops of distilled water.

At the pH of the boric acid (4.2) the indicator is a faint pink, which turns blue at a more alkaline pH, in this case caused by the ammonia. The boric acid solution is then titrated back to its original pH with the aid of a capillary micro burette. The tip of the burette is kept beneath the surface of

the liquid, which is stirred by means of a stream of air during this titration. A receiving tube containing the boric acid indicator mixture only serves for matching the color of the end-point.

### *Procedure for Micro-Kjeldahl Analysis*

0.2 ml of digestion mixture is added to the sample to be analyzed, or to 0.1 ml of standard ammonium sulfate solution in a control analysis, in a digestion tube and digested on a hot-plate. After it has cooled for a few moments, the walls of the test-tube are washed down with 1.0 ml of distilled water. From here on the procedure of aeration and titration is continued as described.

Table I shows the results obtained with 10 and 100  $\gamma$  of nitrogen in analyses of standard ammonium sulfate solution.

TABLE I

*Determination of Ammonia Nitrogen in 0.1 ml of Standard (NH<sub>4</sub>) SO<sub>4</sub> Solution*

N found			
N present 10.0 $\gamma$		N present 100.0 $\gamma$	
$\gamma$	$\gamma$	$\gamma$	$\gamma$
10.0	10.2	98.6	99.4
10.0	10.0	98.0	100.0
10.5	9.8	99.4	99.8
9.8	10.0	99.6	99.0
10.0	9.8	100.0	100.0

**Non-Protein Nitrogen**—The protein of 0.1 ml of serum is precipitated with 2.5 ml of trichloroacetic acid and centrifuged for 10 minutes at 2000 R P M. A 1.0 ml aliquot is then digested with 0.2 ml of digestion mixture. After it has cooled for a few moments, 1 ml of distilled water is added and the aeration and titration procedure continued as above.

**Calculation**— $\text{Titration} \times 2600 = \text{mg per cent of non protein N}$

**Total Protein (Total Nitrogen)**—0.1 ml of serum is diluted to 1 ml with distilled water. 0.1 ml of this mixture is digested with 0.2 ml of digestion mixture on a hot-plate. The tubes are placed at an angle of about 45° and care is taken that none of the liquid bumps out. The digestion is finished when the liquid turns colorless or bluish green. It is allowed to cool for a few moments and 1 ml of distilled water is added to wash down the walls of the test-tube. The aeration and titration are carried out exactly as described for known ammonium salts in the presence of the digestion mixture.

A reagent blank is run through simultaneously.

*Calculation*—Total nitrogen, ml of titration  $\times 0.0714 \times 14$  = amount of N in sample, ml of titration  $\times 10,000$  = mg per cent of Kjeldahl N

Total protein, (mg per cent of total N minus mg per cent of non-protein N)  $\times 6.25/1000$  = gm per cent of total protein

*Albumin*—The globulin of 0.2 ml of diluted serum (0.1 in 1.0 ml) is precipitated with 0.3 ml of sodium sulfate in a small test-tube (100  $\times$  12 mm) and shaken up with 1 ml of ether for 1 to 2 minutes. The tubes are then centrifuged for 10 minutes at 2000 R P M, 0.2 ml aliquots of the aqueous layer are digested with 0.2 ml of digestion mixture, and the Kjeldahl nitrogen determined as described before.

*Calculation*—Titration  $\times 12,500$  = mg per cent of albumin N + non protein N, (mg per cent of albumin N + non-protein N minus mg per cent of non-protein N)  $\times 6.25/1000$  = gm per cent of albumin

TABLE II

*Determination of Nitrogen by Micro-Kjeldahl and Authors' Ultramicromethod in Blood Serum*

The values are expressed in mg per 100 ml of serum

Non protein N		Total N		Albumin N	
Kjeldahl	Authors	Kjeldahl	Authors	Kjeldahl	Authors
33.4	33.1	801	793	556	560
33.6	33.3	835	825	583	587
34.3	35.1	924	925	593	583
34.5	34.8	946	932	608	604
37.6	38.0	1024	1018	614	612
39.2	39.0	1079	1079	716	706

*Results*—Table II shows the close agreement obtained when the authors' ultramicromethod was compared with the micro-Kjeldahl method (2, 4)

#### *Procedure for Urea*

0.1 ml of serum, spinal fluid, or urine providing a sample with 10 to 200  $\gamma$  of urea N, is added to the contents of a digestion tube which consists of 1 drop of phosphate buffer, 1 drop of urease extract, and 3 drops of antifoam reagent. After an incubation period of 10 minutes 0.5 ml of potassium carbonate is added, and the ammonia is aerated for 20 to 30 minutes. Urines are aerated a little longer because of the high values of urea. Otherwise, 0.1 ml of urine may first be diluted to 1 ml with distilled water, and then 0.1 ml of the mixture treated in the same manner as serum. For the titration a capillary micro burette is employed with 0.0714 N sulfuric acid to simplify the calculation.

*Calculation*—Mg of urea N per 100 ml of sample = (amount of urea N  $\times$  100)/  
(ml of sample) = ml of acid  $\times$  1000 when 0.0714 N acid is employed

*Results*—Table III shows the results obtained with 10, 100, and 200  $\gamma$  of urea N. It may be noticed that the absolute error was larger with the ordinary 1 ml micro burette than with the capillary micro burette

TABLE III  
*Determination of Urea Nitrogen in 0.1 Ml of Standard Urea Solution*

Standard I * 10.0 $\gamma$ urea N present	Standard II † 100 $\gamma$ urea N present	Standard III † 200 $\gamma$ urea N present
$\gamma$	$\gamma$	$\gamma$
10.0	100	200
10.0	100	200
10.0	96	202
10.2	100	200
10.0	100	200
10.0	102	202
10.0	100	202
10.2	100	200
10.0	98	198
9.8	100	200

\* Capillary micro burette

† Ordinary 1 ml micro burette

TABLE IV  
*Comparison of Urea Nitrogen Values Determined on 1.0 Ml of Serum with Those Determined on 0.1 Ml of Serum*

The results are expressed in mg per 100 ml

Micromethod	Ultramicromethod	Micromethod	Ultramicromethod
17.2	17.2	56.4	56.5
16.6	16.6	18.0	17.8
18.2	18.2	10.0	10.2
28.4	28.5	14.2	14.5
14.4	14.3	21.2	21.6

Table IV shows the results obtained with 1.0 ml of serum by the method of Sobel, Yuska, and Cohen (1) and those obtained by the authors' method with 0.1 ml of serum. The excellent agreement reached establishes the validity of the new method.

In Table V results are shown which were obtained when known amounts of urea were added to 0.1 ml of serum. The amounts of urea N recovered were very close to the theoretical amounts added, the error varying between 0 and 0.4 per cent.

Table VI shows the average recovery of 100 urea nitrogen determinations with standard solutions and with known amounts of urea added to 0.1 ml of serum. These values were obtained under daily routine conditions and not by the investigators. The low standard deviation obtained under these conditions indicates a high degree of precision even under routine conditions.

## DISCUSSION

It is important to remove impurities from newly obtained rubber stoppers and tubing. This is accomplished by boiling in a dilute solution of

TABLE V  
*Recovery of Urea Nitrogen Added to 0.1 Ml of Serum*

100.0  $\gamma$  added to each sample

The values are expressed in micrograms of urea N

Present in serum	Recovered	Present in serum	Recovered
17.2	100.0	56.5	99.8
16.6	100.0	17.8	100.3
18.2	100.2	10.2	100.0
28.5	100.0	14.5	100.0
14.3	99.6	21.6	100.2

TABLE VI

*Recovery of Urea Nitrogen on 100 Routine Determinations (Mean Values)*

The values are expressed in micrograms of urea N

Sample	Added	Recovered	Average deviation	Standard deviation
Standard solution	10.0	10.243	0.1445	0.1940
0.1 ml urea solution added to 0.1 ml serum	100.0	100.186	0.4432	0.6535

sodium hydroxide followed by washing with distilled water and finally with 2 per cent boric acid.

By using a wire basket on a hot-plate thirty Kjeldahl digestions may be carried out simultaneously with no bumping. In addition, the same set-up can be employed to distill off water in the serum or various serum filtrates as a preliminary step to digestion.

The new antifoaming mixture, caprylic alcohol saturated with thymol, employed for the urea estimation probably works because the thymol alters the surface properties of the proteins in addition to the usual behavior of caprylic alcohol in increasing interfacial tension. Caprylic alcohol alone did not reduce foaming sufficiently to be useful. This was also true of a

number of other higher alcohols tested Castor oil did prevent foaming but was not as convenient to handle

The indicator mixture of bromocresol green and methyl red gives a very sharp end-point, the change of the color being from blue to pink, passing a colorless state, which indicates the closeness of the end-point Because of this sharp end-point this indicator is definitely preferable to Patterson's indicator, with which the last change from blue to violet has to be watched very carefully The ratio of 8 parts bromocresol green to 1 part of methyl red was found to be more suitable for our purposes than the proportions recommended by Ma and Zuazaga (2)

The aeration is started slowly and the air regulated with a clamp until the air bubbles rise evenly throughout the system The bubbles should possibly not go up higher than half way to the side arm, otherwise the danger exists that the liquid might be carried over to the next tube In

TABLE VII

*Influence of Diluting Serum 1 10 on Precipitation of Globulin with Sodium Sulfate Kjeldahl N in filtrate, in mg per 100 ml of serum*

	Na <sub>2</sub> SO <sub>4</sub> in final mixture		
	13.8 per cent	13.8 per cent	21.6 per cent
	Diluted serum	Undiluted serum	Undiluted serum
Sample 1	718	895	712
" 2	660	825	658
" 3	676	852	660

\* Treated according to the technique described by the authors for albumin determination

order to obtain a small air bubble easy to regulate, the glass tubing is drawn out to a fine tip

The titration involves no special precautions With practice a titration with a capillary micro burette takes no more time than with an ordinary burette Both the burette tip and the stirring tip are inserted into the liquid to be titrated The stirring mechanism is attached to the micro burette (For our purposes an air stirring device was satisfactory) The air is purified by passing it through a glass column containing calcium chloride and soda lime The tip of the stirring device is a glass tube of small diameter drawn out to a capillary opening The speed of the air passing through the device into the solution is regulated with a clamp

The comparison of the solution with a pure boric acid indicator solution is of help in titrating to the exact end-point The volume of liquid in the matching color tube should be similar to the one of the solution to be titrated

The reagent blank of urea nitrogen is negligible, the one of Kjeldahl nitrogen is of the order of 0.0002 to 0.0006 ml of titration with 0.0714 N acid

The method of albumin estimation recommended requires further explanation. The final concentration of sodium sulfate in the procedure is 13.8 per cent, at which concentration the precipitate is mostly euglobulin and the filtrate pseudoglobulin I, pseudoglobulin II, albumin, and non-protein nitrogen (5). However, it was found that when the serum was first diluted 1:10 with distilled water and 0.3 ml of 23 per cent sodium sulfate was added to 0.2 ml of the diluted serum the precipitate consisted of all the globulins. The results obtained compared well with the usual method, in which 3 ml of 23 per cent sodium sulfate were added to 0.2 ml of serum. The experimental proof for the validity of our method of albumin determination is given in Table VII. When undiluted serum was added to sodium sulfate, higher results were obtained in the filtrate of a solution containing 13.8 per cent sodium sulfate than with 21.6 per cent sodium sulfate. This observation is in agreement with that of previous workers (5). However, the addition of diluted serum to 23 per cent sodium sulfate so as to produce a solution which is 13.8 per cent with respect to sodium sulfate gave results similar to those usually obtained with 21.6 per cent sodium sulfate.

Apparently the properties of globulins are changed when serum is diluted with distilled water, so that they precipitate more readily than they would otherwise. Though the globulins are known to be insoluble in water and albumins are soluble, one observes a cloudiness but not a precipitate on diluting serum, probably due to protective colloids present in the serum.

#### SUMMARY

A simple titrimetric ultramicromethod employing aeration is described for urea nitrogen, total protein, albumin, and non-protein nitrogen in blood serum and other nitrogenous compounds. The method is rapid and a large number of determinations may be carried out simultaneously.

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# THE ACTION OF "ALKALINE" PHOSPHATASE

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Certain groups have been found to be closely related to the specific activities of many biologically active proteins. While there is little question that the total specific activity is frequently related to the integrity of the molecule as a whole, the destruction or alteration of free primary amino groups, probably of lysine, the sulfhydryl groups of cysteine, and the phenolic hydroxyl groups of tyrosine have been found to parallel the loss in activity in many cases.

The nature of the active groups in "alkaline" phosphomonoesterase should be of particular interest in view of the unusually high pH optimum, 8.5 to 10.0, for enzymic hydrolysis and in view of the relatively low molecular weight of the molecule (1).

In contradistinction to adenosinetriphosphatase the sulfhydryl group has been shown not to be involved in phosphomonoesterase activity (2). Sizer (3) in a study of beef intestinal phosphatase has suggested that the phosphatase activity may be related to unaltered tyrosine in the molecule. This is based on the inactivation of phosphatase by dilute iodine (which has been confirmed in the present investigation) or permanganate, and the observation that there is a concomitant shift in the ultraviolet absorption spectrum characteristic of an alteration in the tyrosine component of the enzyme preparation. The possibility of more than a single group playing a rôle in enzyme activity cannot be neglected. Li *et al.* (4) have found that both the tyrosine and amino groups are essential for the full activity of the lactogenic hormone.

Many compounds are known to act specifically with groups in the protein molecule without alteration or disruption of the molecule as a whole. In the present investigation the significance of the amino and tyrosine groups in phosphatase have been studied by ketene acetylation of amino and phenolic OH groups, by blocking of amino groups with formaldehyde or by reaction with phenyl isocyanate, and by deamination with nitrous acid.

## EXPERIMENTAL

*Enzyme Preparations*—Purified beef intestinal phosphatase was kindly supplied to us by Dr. Gerhard Schmidt. The enzyme which was extremely active (1 mg. hydrolyzed 40 mg. of phosphorus as phosphate from

sodium  $\beta$ -glycerophosphate in 15 to 20 minutes at 37°) was prepared from a tryptic digest of calf intestinal mucosa by ammonium sulfate precipitation and alumina adsorption and desorption (5). The material is stable in solution but very sensitive to increased hydrogen ion concentration and therefore could not be effectively subjected to acid treatment.

*Crude phosphatase preparations* were made by grinding guinea pig tissues (bone, kidney) in chloroform water and allowing the brei to autolyze by standing at room temperature for 24 hours. The digests were then centrifuged and the clear supernatants were used as the enzyme preparations.

These preparations are quite stable, retaining activity for several days, and are not particularly sensitive to increased hydrogen ion concentration.

*Phosphatase Estimation*—Phosphatase was estimated essentially by the micromethod of Shinowara, Jones, and Reinhart (6). The substrate at pH 9.6 contained 0.5 per cent sodium  $\beta$ -glycerophosphate (Eastman Kodak) in 0.02 M sodium diethyl barbiturate (Merck) and 0.0021 M  $MgCl_2$ . Incubation was carried out in a thermoregulated water bath at 37° for 20 minutes with the intestinal phosphatase and for 1 hour with the crude preparations. The liberated phosphate was estimated photocolormetrically with a Hilger absorptiometer.

*Acetylation with Ketene* (7)—Ketene has been used for the acetylation of pepsin (8), insulin (9), parathyroid hormone (10), pituitary hormones (11), and amylase (12). Ketene reacts with primary and secondary amines, sulfhydryl groups, and the phenolic hydroxyl group of tyrosine, not with guanidino groups or aliphatic hydroxyl groups (13). The reaction with primary amines is the most rapid (9), that with the tyrosine relatively slow.

A sample of the highly purified beef intestinal phosphatase was treated with freshly distilled ketene according to the method of Herriott and Northrop (8) at pH 8.0 and at 0°. The pH was adjusted carefully throughout and the samples withdrawn at intervals as indicated in Fig. 1. The samples were diluted and dialyzed overnight, and then tested for phosphatase activity in the usual manner. Controls were run in exactly the same manner, except that they were not treated with ketene.

The relatively rapid decrease in phosphatase activity would indicate that the loss is not due to the acetylation of phenolic groups of tyrosine alone, which would have been acetylated much more slowly. The rate of inactivation of phosphatase by ketene is practically identical with the rate observed by Little and Caldwell (12) for pancreatic amylase. These authors found that 15 minutes acetylation, causing a loss of 78 per cent of the activity, resulted in a loss of only 13 per cent of the tyrosine groups. That the activity is probably not related alone to the tyrosine groups in the molecule is further strengthened by the fact that no appreciable activity was recovered when inactive acetylated intestinal phosphatase was sub

jected to hydrolysis at pH 11, conditions found to be suitable for the deacetylation of acetylated pepsin (8), parathyroid hormone (10), and insulin (9). Herriott and Northrop (8) found that the conditions for deacetylation of acetylated phenolic hydroxyl groups were without effect on acetylated amino groups.

*Treatment with Phenyl Isocyanate*—Phenylureido derivatives of serum albumin, plastin, and tobacco mosaic virus have been prepared by treatment with phenyl isocyanate. Hopkins and Wormall (14) have concluded that phenyl isocyanate reacts only with the free amino groups of the lysine

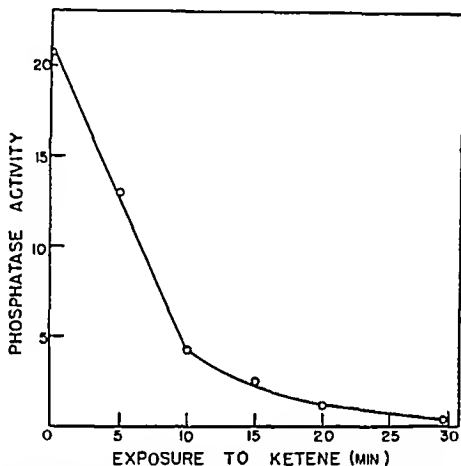


FIG 1 Ketene inactivation of purified intestinal "alkaline" phosphatase as a function of the length of exposure to ketene. Phosphatase activity is expressed in units each of which is equal to the liberation of 1 mg of phosphorus as phosphate from sodium  $\beta$ -glycerophosphate in 20 minutes at 37° by 100 cc of the diluted enzyme preparation.

components of the protein under very mild reaction conditions (0° and pH 8.0) and that further damage of the protein does not occur.

Samples (4.5 cc) of purified beef intestinal phosphatase as well as crude extracts of guinea pig bone and kidney phosphatase were treated with pure phenyl isocyanate which was added over a period of 2 to 4 hours at 0° and the reaction mixture was kept adjusted to pH 8.0. The solid precipitate of diphenylurea was centrifuged off and the solution dialyzed overnight. Controls were run in every case without the phenyl isocyanate. The results of typical experiments are given in Table I.

It is obvious that treatment with phenyl isocyanate largely eliminates the phosphatase activity of both the highly purified preparation and the

*Treatment with Formaldehyde*—The reaction of amino groups with formaldehyde is well known and has been extensively studied (16). Samples of the highly purified intestinal phosphatase and of the crude phosphatase extracts were treated under varying conditions of formaldehyde concentration, time of reaction, and hydrogen ion concentration. All reaction mixtures were dialyzed for 18 hours to remove formaldehyde before phosphatase was estimated. Controls were similarly treated. It can be seen from Table II that there is a pronounced decrease in activity after formaldehyde treatment. The considerably more extensive inactivation of the bone and kidney phosphatase as compared to the purified intestinal phosphatase is striking, since it was found that ketene and phenyl isocyanate inactivate the purified enzyme so completely. This suggests that the extensive inactivation of the crude preparations may be due to an effect of formaldehyde on proteins associated with phosphatase, that groups in the intestinal phosphatase other than free amino groups which react with isocyanate and ketene but not with formaldehyde contribute to the activity, or that the amino groups in the intestinal preparation are not as easily available to the formaldehyde. It appears not unlikely that there may be an inherent difference between the intestinal phosphatase on the one hand and the bone and kidney phosphatase on the other. Bodansky (17) has suggested previously, on the basis of bile salt inhibition studies, that there is such a difference, the former being little affected by bile salts, while the latter are strongly inhibited.

#### SUMMARY

Preparations of intestinal, bone, and kidney phosphatase have been studied by treatment with ketene, phenyl isocyanate, nitrous acid, and formaldehyde and appear to resemble the parathyroid hormone (10), diphtheria toxin (18), certain pituitary hormones (11), gonadotropic hormones of pregnant mare serum (11), and amylase (12) in that the intact amino group is apparently essential for full enzymic activity. In this respect the phosphatase differs from pepsin (8), insulin (9), human chorionic gonadotropic hormone (11), and tobacco mosaic virus (19) whose activities are largely independent of the presence of amino groups. On the other hand, the phosphatase resembles the latter substances mentioned in that it appears to require the intact phenolic hydroxyl group for its normal activity.

Phosphatase is therefore similar to the lactogenic hormone (5) which is the only other biologically active substance reported to require both the intact amino and phenolic hydroxyl groups for the unfolding of its complete activity.

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*Treatment with Formaldehyde*—The reaction of amino groups with formaldehyde is well known and has been extensively studied (16). Samples of the highly purified intestinal phosphatase and of the crude phosphatase extracts were treated under varying conditions of formaldehyde concentration, time of reaction, and hydrogen ion concentration. All reaction mixtures were dialyzed for 18 hours to remove formaldehyde before phosphatase was estimated. Controls were similarly treated. It can be seen from Table II that there is a pronounced decrease in activity after formaldehyde treatment. The considerably more extensive inactivation of the bone and kidney phosphatase as compared to the purified intestinal phosphatase is striking, since it was found that ketene and phenyl isocyanate inactivate the purified enzyme so completely. This suggests that the extensive inactivation of the crude preparations may be due to an effect of formaldehyde on proteins associated with phosphatase, that groups in the intestinal phosphatase other than free amino groups which react with isocyanate and ketene but not with formaldehyde contribute to the activity, or that the amino groups in the intestinal preparation are not as easily available to the formaldehyde. It appears not unlikely that there may be an inherent difference between the intestinal phosphatase on the one hand and the bone and kidney phosphatase on the other. Bodansky (17) has suggested previously, on the basis of bile salt inhibition studies, that there is such a difference, the former being little affected by bile salts, while the latter are strongly inhibited.

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Phosphatase is therefore similar to the lactogenic hormone (5) which is the only other biologically active substance reported to require both the intact amino and phenolic hydroxyl groups for the unfolding of its complete activity.

# USE OF THE SPECTROPHOTOMETER IN THE DETERMINATION OF CYSTINE BY SULLIVAN'S REACTION\*

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Several methods have been used for the determination of cystine. Of the different types of reactions proposed, that of Sullivan is generally considered to be the most specific. The original reaction was proposed for the determination of cystine (1), but was later adapted to the determination of cystine (2). Modifications of the original procedure of Sullivan have been proposed by Prunty (3), Lugg (4), and Rossouw and Wilken-Jorden (5). The elimination of interfering substances by precipitation of cystine as the cuprous mercaptide (6-8) is an improvement for the use of Sullivan's reaction for determining cystine in acid hydrolysates.

Bushill, Lampitt, and Baker (9) used the Zeiss photometer to study some of the factors influencing the color produced by the Sullivan reaction and some of its modifications. The introduction of the photoelectric colorimeter has provided a tool for increasing the simplicity and accuracy of colorimetric determinations. It also has provided a means of quantitatively studying the different factors influencing the intensity of color produced in a given reaction. It therefore seemed desirable to adapt the Sullivan reaction for use with the spectrophotometer.

## EXPERIMENTAL

A Coleman spectrophotometer, type 10-S, was used in obtaining the data presented in this paper. Rossouw and Wilken-Jorden's modification (5) of Sullivan's reaction for cystine was used. The color was developed as outlined in this method on a solution containing no cystine (blank) and on one containing 150 parts per million of cystine. Per cent transmittance was determined between the wave-lengths of 400 and 900  $m\mu$  on both colored solutions with water as the reference solution. The two curves obtained are presented in Fig. 1. A curve was obtained similarly by determining the per cent transmittance on the treated solution containing 150 p.p.m. of cystine, when the spectrophotometer was balanced against the treated blank (Fig. 1). The greatest difference in per cent transmittance between the treated cystine solution and the treated blank occurred at a wave-length between 490 and 500  $m\mu$ .

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*Influence of Time between Preparing Reagent Solutions and Use on Per Cent Transmittance (Table I)*—The most satisfactory results were obtained when the reagents were all prepared just before use. However, it was not absolutely necessary that they should all be prepared then. When all of the reagent solutions were allowed to stand before being used, there was a continuous increase in color in the blank containing no cystine. The color

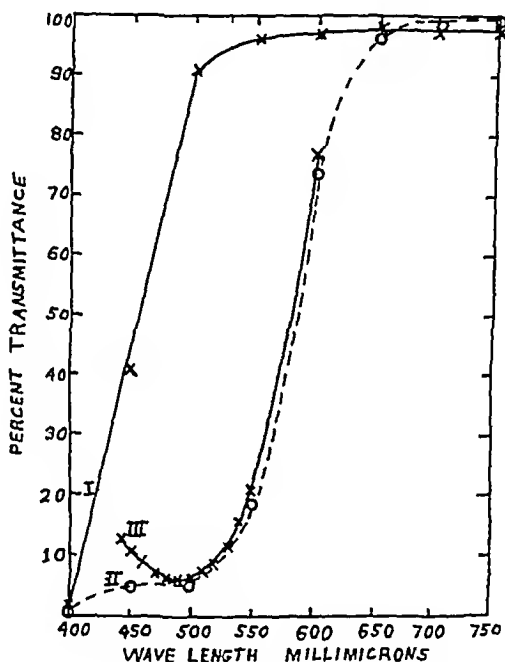


FIG 1 Per cent transmittance plotted against wave-length for solutions prepared by Sullivan's reaction for cystine. Curve I was obtained with treated water when the spectrophotometer was balanced against water. Curve II was obtained with a treated solution containing 150 p p m of cystine with water as a reference solution. Curve III was obtained with a treated solution containing 150 p p m of cystine, with treated water as a reference solution.

increased after the 1st hour of standing in the solutions containing cystine, but after 5 hours there was a considerable decrease in color at all concentrations of cystine used.

Further investigation showed that there was little difference in the color developed whether the NaCN or the Na<sub>2</sub>SO<sub>3</sub> were prepared fresh or allowed to stand for as long as 5 hours before use. The sodium 1,2-naphtho-

quinone-4-sulfonate (color reagent) should not be allowed to stand much longer than an hour before use, and the  $\text{Na}_2\text{S}_2\text{O}_4$  must be prepared just before being used (Table I)

*Influence of Acidity of Cystine Solution on Per Cent Transmittance*—The necessity of having the acidity carefully adjusted is evident from Table II. Considerable error would result from having the acidity of the

TABLE I

*Influence of Time between Preparation of Reagent Solutions and Their Use on Color Produced by Sullivan Reaction for Cystine*

Reagents not prepared just before use	Cystine content of solution	Time between preparing and using reagents when not prepared just before use		
		0 hr	1 hr	2 hrs
	<i>p p m</i>	<i>per cent transmittance</i>	<i>per cent transmittance</i>	<i>per cent transmittance</i>
All	0	90.5	89.5	71.0
"	50	24.0	22.0	34.0
"	100	5.0	3.5	14.0
"	150	3.0	1.5	10.0
Sodium 1,2-naphthoquinone-4-sulfonate	50	28.4	28.2	26.8
$\text{NaCN}$	50	28.4	28.8	29.0
$\text{Na}_2\text{SO}_3$	50	28.4	29.0	28.4
$\text{Na}_2\text{S}_2\text{O}_4$	50	28.4	24.8	38.8

TABLE II

*Influence of Acidity of Cystine Solution on Color Produced by Sullivan Reaction for Cystine (50 P p m of Cystine)*

Acidity	pH	Per cent transmittance
<i>N</i>		
0.10	1.10	27.0
0.08	1.23	25.5
0.06	1.36	23.5
0.04	1.54	21.5
0.02	1.80	20.0

unknown differ from that of the standards. Sullivan (2) in his original procedure calls for adjusting the pH of the unknown solution to about 3.5 and then making to volume with 0.1 N HCl. High results were obtained in the present study when a solution prepared in this way was compared with a standard cystine solution containing 0.1 N HCl. Better results were obtained by adjusting the pH of the unknown to 1.0 with a pH meter, and then making to volume with 0.1 N HCl.

*Influence of Time between Adding Different Reagents on Per Cent Transmittance*—Rossouw and Wilken-Jorden (5) found that the most intense color was obtained when the  $\text{Na}_2\text{SO}_3$  was added 20 to 30 seconds after the sodium 1,2-naphthoquinone-4-sulfonate. The time between the additions of the other reagents also must be very carefully controlled. The data presented in Table III show the influence a difference of 5 minutes had on the final color developed. Ten samples can be satisfactorily run at once by allowing 60 seconds between samples for the addition of a reagent. In this way exactly 10 minutes can elapse between the addition of the NaCN and the color reagent, 20 to 30 seconds between the color reagent and  $\text{Na}_2\text{SO}_3$ , and 30 minutes between the  $\text{Na}_2\text{SO}_3$  and the  $\text{Na}_2\text{S}_2\text{O}_4$ .

*Influence of Time of Color Reading after Adding Sodium Hydrosulfite on Per Cent Transmittance*—The solution should stand between 10 and 40

TABLE III

*Influence of Time between Adding the Different Reagents on Color Produced by Sullivan Reaction for Cystine (Solution Contained 50 P p m of Cystine)*

Between reagents given below	Time between adding reagents	Per cent transmittance
	min	
NaCN and sodium 1,2-naphthoquinone-4-sulfonate	5	26.5
	7	27.5
	10	30.0
$\text{Na}_2\text{SO}_3$ and $\text{Na}_2\text{S}_2\text{O}_4$	20	30.5
	25	28.5
	30	27.0
	35	26.0
	40	25.0

minutes after the  $\text{Na}_2\text{S}_2\text{O}_4$  is added before reading the color (Fig. 2). The color intensity was greatest during the first 10 minutes after developing the color, but it decreased rapidly during this time. It was fairly constant during the next 30 minutes, or until 40 minutes after adding the  $\text{Na}_2\text{S}_2\text{O}_4$ . Thereafter the color again gradually decreased in intensity.

*Suggested Procedure for Determination of Cystine in Solution*—Adjust pH of solution to 10 and make to volume with 0.1 N HCl. To 5 ml of the solution containing 25 to 150 p p m of cystine add 2 ml of freshly prepared 10 per cent NaCN solution, shake, and allow to stand exactly 10 minutes. Add 1 ml of freshly prepared 0.5 per cent solution of sodium 1,2-naphthoquinone-4-sulfonate, shake, and let stand 20 to 30 seconds. Add 5 ml of freshly prepared 10 per cent solution of  $\text{Na}_2\text{SO}_3$  in 0.5 N NaOH, shake, and allow to stand exactly 30 minutes. Add 1 ml of freshly prepared 2 per cent solution of  $\text{Na}_2\text{S}_2\text{O}_4$  in 0.5 N NaOH, shake, and allow to stand for 10

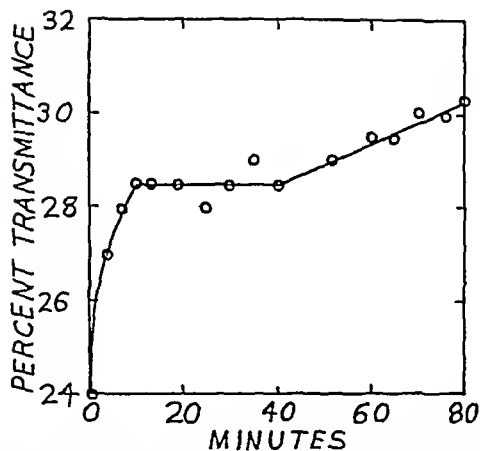


FIG 2 Per cent transmittance plotted against time between the addition of the  $\text{Na}_2\text{S}_2\text{O}_4$  and reading the color The original solution contained 50 p p m of cystine

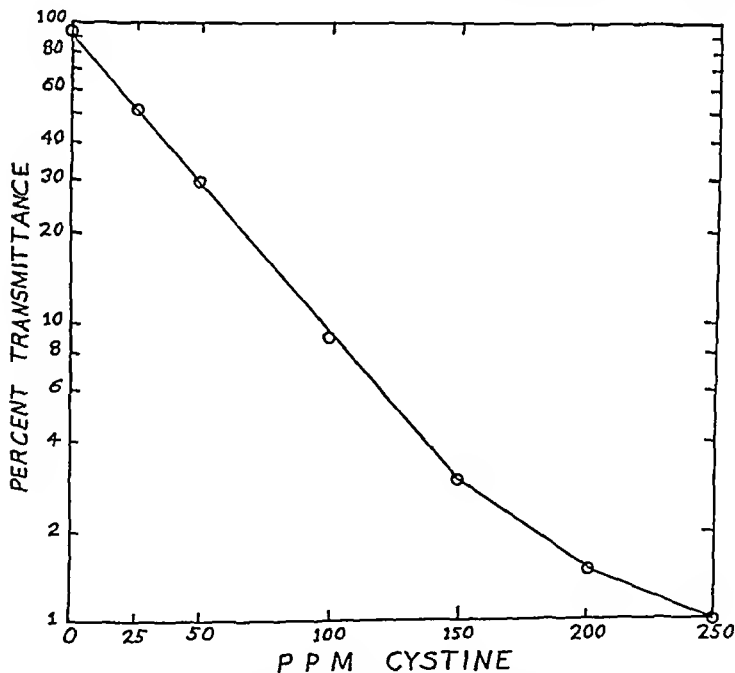


FIG 3 Typical standardization curve for Sullivan's reaction for cystine The spectrophotometer used had a  $30 \text{ m}\mu$  slit and was set at a wave-length of  $500 \text{ m}\mu$  The p p m of cystine in the original solution are plotted against the log of the per cent transmittance

to 40 minutes. Read the per cent transmittance in the spectrophotometer at a wave-length of 500  $m\mu$ . Prepare a standardization curve by plotting on semilog paper the per cent transmittance against the cystine content of known solutions. Read the cystine content of the unknown solution from this standardization curve.

A typical standardization curve is presented in Fig 3. New standardization curves should be prepared every few weeks, since there may be a considerable change from time to time, the causes of which are not yet known. For example, the data in the first part of Table I were obtained a year before those in the second part. The standardization curves obtained at these times were quite different. These differences may be observed at other places in the data here presented. The curve in Fig 3 is a straight line from 0 to 150 p p m of cystine. After that it levels off considerably. This is characteristic of all the curves obtained, and places the limit for greatest accuracy between these levels.

#### SUMMARY

The Sullivan reaction for the determination of cystine has been studied and the procedure adapted for use with the spectrophotometer. Maximum differences in transmittance between a blank and a cystine solution treated by this procedure were obtained at wave-lengths of 490 to 500  $m\mu$ . Most consistent results were obtained when the solutions contained between 25 and 150 parts per million of cystine, when they were adjusted to a pH of 1.0 with a pH meter, when all reagent solutions were freshly prepared before use, when the time between adding the reagents was held constant, and when the color reading was made between 10 and 40 minutes after the last reagent was added.

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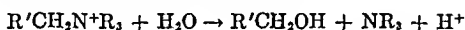
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## LETTERS TO THE EDITORS

### THE FORMATION OF HYDROGEN ION IN THE CLEAVAGE OF THIAMINE BY THE FISH PRINCIPLE

Sirs

The enzymatic cleavage of thiamine by the Chastek paralysis factor of fish viscera yields 4-methyl-5-hydroxyethylthiazole and 2-methyl-4-amino-5-hydroxymethylpyrimidine<sup>1</sup>. The over-all reaction is thus the hydrolytic cleavage of a carbon to quaternary nitrogen linkage with the addition of the hydroxyl group to the methylene substituent in the pyrimidine and the conversion of the quaternary nitrogen to a tertiary nitrogen. This is summarized in the following representation



If a hydrogen ion is produced, the reaction should yield carbon dioxide when carried out in bicarbonate buffer. The hypothesis would therefore be capable of test with the manometric technique.

With the usual Warburg apparatus, an extract equivalent to 75 or 100 mg of acetone-desiccated carp viscera<sup>2</sup> was allowed to react for 2 hours at 37.5° in an atmosphere of air with 10 micromoles of thiamine in a total volume of 3 ml of 0.2 M sodium bicarbonate (pH 7.4) in 10 per cent sodium chloride. Manometric readings were taken at 15 minute intervals, and at the end of the experimental period the contents of the vessels were analyzed for the thiamine remaining by either the Melnick-Field or thiochrome procedures. The carbon dioxide produced (corrected for control flasks) in three different experiments is recorded in the table.

Experiment No	1	2	3
Carbon dioxide, micromoles	8.30	5.40	5.40
Thiamine destroyed, micromoles	9.70	9.56	8.81
$k \times 10^3$ (1st order), average	14.6	6.80	6.00

From the summary it is obvious that the action of the fish principle on thiamine results in the release from bicarbonate buffer of extra carbon

<sup>1</sup> Krampitz, L. O., and Woolley, D. W., *J. Biol. Chem.*, **152**, 9 (1944).

<sup>2</sup> Sealock, R. R., Livermore, A. H., and Evans, C. A., *J. Am. Chem. Soc.*, **65**, 935 (1943).

dioxide which in these experiments was equivalent to 85.7, 56.5, and 61.3 per cent, respectively, of the thiamine destroyed as determined by chemical analysis. Therefore, it may be concluded that a hydrogen ion is produced in the enzymatic reaction. Since the hydrogen ion may arise from the water molecule involved in the formation of the alcohol, it is suggested that the fish principle is unique among those enzymes catalyzing hydrolytic reactions. Discussion of this and other possibilities is reserved for a more detailed communication. However, it may be concluded that the manometric procedure furnishes an additional tool for the investigation of the enzyme reaction.

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# CHANGES IN THE ELECTROPHORETIC COMPOSITION OF CRYSTALLINE HEN'S EGG ALBUMIN WITH AGE

Sirs

It has been shown<sup>1</sup> that hen's egg albumin (Ea) has two electrophoretic components, A<sub>1</sub> and A<sub>2</sub>, in the pH range 5 to 10. The relative amount of A<sub>2</sub> was found to vary between 12 and 25 per cent.

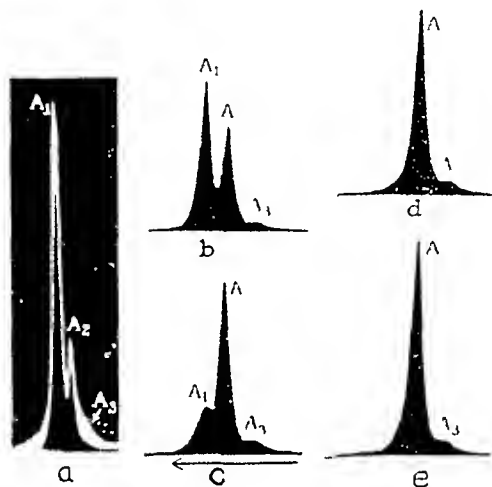


FIG 1 Electrophoretic patterns of egg albumin (a) Ea V, freshly prepared, (b) Ea V aged 6 months at 4-6° as salt free solution with toluene as preservative, (c) Ea III aged 1 month as salt free solution, (d) Ea III aged 7.5 months as salt free solution, (e) Ea III aged 7.5 months as salt free solution plus 11 months in 0.1 M phosphate buffer at pH 6.8

Several preparations of hen's egg albumin made in these laboratories by two different methods,<sup>2,3</sup> and stored either as powders or as isoelectric salt-free solutions, were examined electrophoretically at various times. As shown in Fig 1 and the table, component A<sub>2</sub> increased with the age of the preparation, and was usually the only component present after 1 year.

<sup>1</sup> Longworth, L. G., Cannan, R. K., and MacInnes, D. A., *J. Am. Chem. Soc.*, **62**, 2580 (1940).

<sup>2</sup> Heidelberger, M., *An advanced laboratory manual of organic chemistry*, New York (1932).

<sup>3</sup> Kekwick, R. A., and Cannan, R. K., *Biochem. J.*, **30**, 232 (1926).



Conditions of storage	Age	A	Ionic strength	pH	Mobility $\times 10^5$	
					A <sub>1</sub>	A <sub>2</sub>
Ea Preparation III made according to foot-note 2	mos.	per cent				
Salt free isoelectric solution preserved with thymol and toluene	1	77	0.1	6.8 P*	-6.0	-5.4 (e)†
Same, recrystallized from sodium sulfate	3	97	0.02	6.2 A		-5.4
Original salt free isoelectric soln	7.5	100	0.1	6.8 P		-5.4 (d)
Ea Preparation V made according to foot note 2						
Salt free isoelectric solution of Ea preserved with toluene	0.25	21	0.02	6.2 A	-6.5	-5.5 (a)
Same	6	43	0.1	6.8 P	-5.9	-5.1 (b)
"	12	100	0.2	6.8 "		-4.8
Ea Preparation IX made according to foot note 3						
Dry powder	1	14	0.2	6.8 "	-5.3	-5.0
" "	12	20‡	0.2	6.8 "	-5.4	-5.0

\* P = phosphate, A = acetate buffer

† See the section of Fig. 1 indicated

‡ Ea Preparation VI made according to foot-note 3 and aged 14 months as a dry powder contained 100 per cent A<sub>2</sub>

The rate at which A<sub>2</sub> increased was variable but was faster when the preparation was stored in solution. Preparations which contained 100 per cent A<sub>2</sub> were not insoluble at the isoelectric point and were easily recrystallized.

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## A FLAVOPROTEIN ENZYME

Sirs

We have recently described the preparation and properties of an enzyme from rat kidneys which oxidizes *l*-amino acids<sup>1</sup>. The generous cooperation of several pharmaceutical firms in providing us with some 50 kilos of frozen rat kidneys has made it possible to undertake the isolation of the enzyme. From 5 kilos of rat kidney we have obtained by successive salt fractionations about 1 gm of a flavoprotein which oxidizes both dihydrocoenzyme I and *l*-amino acids. Electrophoretic examination at pH 7.4 showed a single component, whereas in the ultracentrifuge two colored components appeared, a heavier fraction composing about 60 per cent of the total protein ( $S_{20} = 13.5$ ) and a lighter fraction (40 per cent,  $S_{20} = 5.0$ ).

By repeated fractionation in the ultracentrifuge a solution of the lighter fraction was prepared which contained less than 5 per cent of the heavy fraction. This isolated light fraction had the same enzymatic activities towards dihydrocoenzyme I and *l*-amino acids as both the original material and solutions containing predominantly the heavy fraction.

The diffusion constant of the light material ( $S_{20} = 5.0$ ) was found to be  $D_{20} = 4.0 \times 10^{-7}$  sq cm per second. Assuming a partial specific volume of 0.75, a molecular weight of about 120,000 was calculated. The average diffusion rate (calculated from the width of the boundary pattern at the inflection point) for the original unseparated material was found to be  $3.0 \times 10^{-7}$  sq cm per second. With  $S_{20} = 13.5$  and  $D_{20} = 3.0$ , a molecular weight of 430,000 was calculated. The value for  $D_{20}$  is erroneously low because of the presence of the lighter component. Since the flavin content is 0.46 per cent (expressed as flavin phosphate), corresponding to a minimum molecular weight of about 100,000, it is believed that the light fraction contains one flavin group per molecule and the heavy fraction four flavin groups per molecule.

The flavin enzyme of the rat kidney resembles in some respects a flavin enzyme previously isolated from cow's milk<sup>2</sup>.

Source of enzyme	Rat kidney	Cow's milk
Substrates of enzyme	<i>l</i> -Amino acids, dihydrocoenzyme I	Purines, aldehydes, dihydrocoenzyme I
No. of flavins per molecule	1 or 4	2 (Probably)
Sedimentation constant	5.0 or 13.5	12.6
% of total color bleached by Na <sub>2</sub> O <sub>4</sub>	66	60

<sup>1</sup> Blanchard, M., Green, D. E., Nocito, V., and Ratner, S., *J. Biol. Chem.*, **155**, 421 (1944).

<sup>2</sup> Corran, H. S., Dewan, J. G., Gordon, A. H., and Green, D. E., *Biochem. J.*, **33**, 1694 (1939). Ball, E. G., *J. Biol. Chem.*, **128**, 51 (1939).

Both flavoproteins have multiple catalytic functions and both can oxidize dihydrocoenzyme I. Also both contain a colored group in addition to flavin which is not bleached by  $\text{Na}_2\text{S}_2\text{O}_4$ .

In the presence of either *L*-leucine or dihydrocoenzyme I, the yellow color and greenish fluorescence of the flavoprotein of rat kidney are bleached almost instantaneously. Shaking the leuco enzyme with air restores the original color. The mechanism of enzyme action thus involves a catalytic cycle of reduction by the substrate and oxidation by molecular oxygen.

The prosthetic group of the enzyme can be split off from the protein by heat or acid treatment. It shows the three characteristic absorption bands of the flavin compounds at 270, 370, and 445  $\text{m}\mu$  respectively. While the chemical properties of the prosthetic group suggest that it is phosphorylated, identity with flavin adenine dinucleotide has been excluded.

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# THE CHOLINE-CONTAINING AND NON-CHOLINE-CONTAINING PHOSPHOLIPIDS OF PLASMA

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The relative amounts of choline-containing<sup>1</sup> (lecithins and sphingomyelins) and non-choline-containing phospholipids (cephalins) in human plasma have been measured by several investigators (1-6). Their results are summarized in Table I. Although as much as 42 per cent of plasma phospholipids has been reported to contain no choline, most workers agree on values between 20 and 30 per cent.

During the course of development of a procedure for the separation of C C from N C C phospholipids (7), it was observed that practically 100 per cent of the phospholipids of dog plasma contains choline. Human plasma was therefore reinvestigated. It is demonstrated here that practically all of the phospholipids found in the plasma of man as well as of dogs are of the C C type.

## EXPERIMENTAL

The plasma used in the present investigation was obtained from sixteen normal human subjects between 20 and 30 years of age, and from five normal dogs. Unless noted otherwise, the samples of blood were removed from the human subjects between 8 00 a m and 9 00 a m, just before breakfast. The dogs had been deprived of food for approximately 16 hours at the time blood was taken from the heart or femoral artery. Heparin was used to prevent clotting. Plasma was separated by centrifugation of the blood sample for 40 to 50 minutes.

*Extraction of Phospholipids*—Two methods were used to extract phospholipids from plasma.

*Direct Method*—Each sample of plasma was pipetted with constant stirring into 25 volumes of a solution consisting of 3 parts of ethanol and 1 part of ethyl ether. The mixture was kept at room temperature for 30 minutes to 2 hours and then filtered. The residue was then extracted with ether for 6 to 12 hours in a Soxhlet apparatus. The ether extract so obtained was added to the alcohol-ether filtrate and the combined extracts concentrated at 50-60° to a small volume (about 1 cc) under reduced pressure in an atmosphere of CO<sub>2</sub>. The phospholipids were extracted from the residue

<sup>1</sup> The terms choline-containing and non-choline-containing have been abbreviated to C C and N C C.

with several portions of warm petroleum ether. The latter was then reduced to a volume of approximately 1 cc by evaporation at 50–60°, and the phospholipids precipitated by the addition of 30 cc of acetone and 5 to 20 drops of a saturated solution of  $MgCl_2$  in absolute alcohol. The mixture was centrifuged and the supernatant discarded. The precipitate was washed with acetone and then dissolved in a mixture of methanol and ether, aliquots of the solution were taken for choline and phosphorus analyses.

*Indirect Method (Colloidal Iron Method)*—According to Folch and aVn Slyke (8), a lipid extract free from nitrogenous contaminants can be obtained by the use of a dialyzed iron solution. Since the presence of such

TABLE I

*Choline-Containing and Non-Choline Containing Phospholipids of Plasma and Serum As Reported by Previous Investigators*

Investigator	Serum or plasma	No of subjects	Phospholipid results					Dietary state of subjects
			Total	Leceithin	Cephalin	Sphingomyelin	Choline phospholipids	
			mg per cent	per cent	per cent	per cent	per cent	
1 Thannhauser <i>et al</i> (1)	Serum	6	226	47	42	11	58	Postabsorptive
2 Erickson <i>et al</i> (2)	Plasma	4	189	52	29	19	71	Not stated
3 Artom (3)	"	16	152		20		80	Postabsorptive
4 Brante (4)	Serum	13	185		22		78	"
5 Bliz (5)	"	2	229		27		73	Not stated
6 Marenzi and Cardini (6)	Plasma	Not stated	204	61	22	17	78	" "

contaminants in a lipid extract might interfere with the determination of choline as it is carried out here, the phospholipids of several samples of plasma were extracted by the procedure described by Folch and Van Slyke. The iron-protein precipitate containing the phospholipids was washed twice with an aqueous salt solution ( $MgSO_4$ ) in order to remove water-soluble contaminants. The precipitate was then extracted twice at room temperature with an alcohol-ether solution. For the first extraction 8 volumes of 1:1 alcohol-ether solution were added and the mixture agitated at intervals for 15 to 20 minutes. After centrifugation, the supernatant was filtered. For the second extraction, 15 volumes of a solution containing 3 parts of alcohol and 1 part of ether were added to the residue and the mixture stirred at intervals for 20 to 30 minutes. This mixture

was then filtered and the filtrate so obtained combined with the first. The combined alcohol-ether extracts were made up to volume. In most cases aliquots of the filtered extract were taken for determinations of choline and phosphorus. In a few instances, however, this alcohol-ether extract was concentrated to a small volume (at 50–60° under reduced pressure and in an atmosphere of CO<sub>2</sub>), the phospholipids reextracted with several portions of warm petroleum ether, and the phospholipids precipitated with acetone and MgCl<sub>2</sub>, as described above. The precipitate was redissolved in a mixture of methanol and ethyl ether, made up to volume, and aliquots of the solution used for the determination of choline and phosphorus.

### *Methods of Analysis and Calculations*

Phosphorus was determined by King's method (9). The factor 25 was used to convert mg of P to mg of phospholipid.

The methods for hydrolysis of phospholipid and determination of choline have been described elsewhere (10).

The molal ratio, choline/P, in phospholipid was calculated from the following expression,

$$\frac{c \times 31}{p \times 139.5}$$

in which  $c$  is the observed mg of phospholipid choline (expressed as the chloride), and  $p$  the observed mg of phospholipid P. The level of C C phospholipids of plasma was calculated by multiplying the mg of total phospholipid per 100 cc of plasma by the molal ratio. The error introduced into this calculation by the slight difference between the average molecular weights of the C C and N C C phospholipids is negligible.

*Test of Choline Method on Plasma Extracts*—The accuracy of the choline method as applied to liver phospholipids has been described previously (10). For the purpose of the present investigation it was necessary to test the choline method on plasma phospholipids. The phospholipids of several samples of plasma were precipitated by acetone and MgCl<sub>2</sub> and washed with acetone. The precipitate was redissolved in a mixture of methanol and ether. An aliquot of each phospholipid solution was taken for the determination of its choline content, to other aliquots were added known amounts of choline and the recoveries of the added choline determined. An average recovery of 101 per cent was obtained (Table II).

### *Results*

Plasma was obtained from sixteen human subjects and five dogs all of which were in the postabsorptive state. The molal ratio, choline/P, was calculated for each sample of plasma phospholipid (Tables III to V). The

ratios are very close to unity for the phospholipids of all plasma samples. This ratio represents the fraction of the phospholipids that contains choline,

TABLE II  
*Recovery of Choline Added to Plasma Phospholipids*

Choline chloride added to sample	Choline initially present*	Total choline found	Recovery of added choline*	
mg	mg	mg	mg	per cent
1 87	3 86	5 84	1 98	106
1 87	2 75	4 64	1 89	101
2 81	2 75	5 62	2 87	102
1 87	2 74	4 71	1 97	105
2 81	2 74	5 57	2 83	101
1 87	1 00	2 87	1 87	100
2 00	0 93	2 88	0 83	94
2 00	1 87	3 80	1 80	96
Average				101

\* All the values are expressed as the chloride

TABLE III  
*Phospholipids of Human Plasma*

All plasma samples were extracted by the direct method

Subject	Phospholipid P	Phospholipid choline	Choline P	Total phospholipids	Choline containing phospholipids	Non-choline-containing phospholipids
	mg per 100 cc plasma	mg per 100 cc plasma	molal ratio	mg per 100 cc plasma	mg per 100 cc plasma	mg per 100 cc plasma
H A	8 06	35 0	0 97	202	196	6
A F	9 76	42 4	0 96	244	234	10
A F	9 42	42 5	1 00	236	236	0
S L	10 0	44 1	0 98	250	245	5
S L	10 2	47 0	1 02	256	256	0
S C	6 88	29 9	0 97	172	167	5
H F	10 3	48 0	1 03	258	258	0
A T †	9 00	37 2	0 92	225	207	18
R R	8 40	36 0	0 95	210	200	10
D K	10 5	46 7	0 99	262	259	3
F G	10 8	47 6	0 98	269	263	5
Average	9 39	41 5	0 98	235	229	6

\* The values are expressed as the chloride

† Blood was removed from subject A T 1 hour after breakfast. All other subjects were in the postabsorptive state at the time blood was taken

provided that each molecule of choline phospholipid contains 1 molecule of choline for each atom of phosphorus. An average ratio of 0 98 for human plasma was found when direct extraction was used and 0 96 when the

TABLE IV

*Comparison of Direct and Colloidal Iron Methods for Extraction of Phospholipids*

Subject	Direct extraction						Colloidal iron extraction					
	Phospholipid P	Phospholipid choline†	Choline P	Total phospholipids	Choline-containing phospholipids	Non-choline containing phospholipids	Phospholipid P	Phospholipid choline†	Choline P	Total phospholipids	Choline-containing phospholipids	Non-choline containing phospholipids
	mg per 100 cc plasma	mg per 100 cc plasma	molal ratio	mg per 100 cc plasma	mg per 100 cc plasma	mg per 100 cc plasma	mg per 100 cc plasma	mg per 100 cc plasma	molal ratio	mg per 100 cc plasma	mg per 100 cc plasma	mg per 100 cc plasma
A T	8 60	39 5	1 02	215	215	0	8 40	36 4	0 94	210	197	13
H B	9 45	41 2	0 97	236	229	7	8 70	36 4	0 93	218	203	15
R K R	8 05	35 2	0 97	201	195	6	7 20	30 8	0 95	180	171	9
A G	9 54	43 5	1 01	238	238	0	9 90	40 2	0 90	248	223	25
W C	9 75	45 2	1 03	244	244	0	8 40	36 7	0 97	210	204	6
J A	5 53	25 5	1 03	138	138	0	4 93	22 4	1 01	123	123	0
C E	11 7	50 0	0 95	293	278	15	10 8	47 1	0 97	271	263	8
L S	9 30	41 6	0 97	232	225	7	8 55	37 4	0 97	214	208	6
Average	8 99	40 2	0 99	225	220	5	8 36	35 9	0 96	209	199	10

\* All subjects were in the postabsorptive state at the time blood was taken

† The values are expressed as the chloride

TABLE V

*Phospholipids of Dog Plasma*

Dog No	Extraction method	Phospholipid P	Phospholipid choline†	Choline P	Total phospholipids	Choline-containing phospholipids
		mg per 100 cc plasma	mg per 100 cc plasma	molal ratio	mg per 100 cc plasma	mg per 100 cc plasma
1	Direct extraction	11 2	51 0	1 01	281	281
2	" "	10 1	47 5	1 05	252	252
3	" "	9 75	45 3	1 03	244	244
4	" "	10 9	50 6	1 03	272	272
4	Colloidal iron	9 20	43 9	1 06	230	230
5	Direct extraction	10 8	51 2	1 05	270	270
5	" " †	10 7	50 2	1 04	268	268
5	Colloidal iron	10 1	47 2	1 04	253	253
5	" " †	9 85	46 2	1 04	246	246

\* All dogs were in the postabsorptive state at the time blood was removed

† The values are expressed as the chloride

‡ Ovalate instead of heparin was used as anticoagulant

colloidal iron extraction method of Folch and Van Slyke was used For dog plasma the respective ratios were 1 02 and 1 05



The amounts of C C and N C C phospholipids were also calculated and recorded in Tables III to V. As was to be expected from the molal ratios noted above, very small amounts of N C C phospholipids, or none at all, were found in plasma.

Slightly lower choline to P ratios were found by the colloidal iron extraction than by direct extraction. The total amounts of phospholipid extracted by the former procedure were also slightly lower.

The levels of plasma phospholipids determined in sixteen human subjects in the postabsorptive state varied from 123 to 293 mg per 100 cc, the average was 215 mg per cent. Not more than 5 per cent of these amounts was of the N C C type. In the five dogs the plasma phospholipids varied from 230 to 281 mg per 100 cc, all were of the C C type. Plasma of dog and man contained from 30 to 50 mg of phospholipid choline per 100 cc (average, 42 mg).

#### DISCUSSION

The results obtained here indicate that practically all of the phospholipids of human and dog plasma contain choline. This observation is not in agreement with the findings of previous investigators (Table I). Brante (4) determined the C C phospholipids of serum of thirteen normal men in the postabsorptive state, he found two values of over 90 per cent for C C phospholipids, but his average value was 78 per cent. Artom studied sixteen subjects in the postabsorptive state (3). His average value for C C phospholipid, namely 80 per cent, is the highest value hitherto reported for human plasma.

It appears unlikely that the discrepancies between the results obtained here and elsewhere can be ascribed to the dietary habits of the subjects studied. It seems more likely that the explanation for the difference is to be found in the methods employed for the analyses of phospholipid choline. Higher choline to phosphorus ratios would be obtained if the choline values found here were excessive. But the following evidence does not support such a view. (1) Satisfactory recoveries of choline were obtained when choline was added to plasma phospholipids (Table II). (2) There was good agreement between the choline to phosphorus ratios obtained by two different extraction procedures, the colloidal iron extraction procedure of Folch and Van Slyke, and the direct alcohol-ether extraction method. In the colloidal iron procedure, the iron-protein precipitate containing the phospholipids was washed twice with an aqueous salt solution *before* extraction of the phospholipids. Such a procedure would be expected to remove free choline (i.e. choline not combined as phospholipids) that may be present in plasma, as well as other substances that may interfere with the measurement of choline.

The method of hydrolysis of phospholipids employed in the present investigation differs from that used by most other workers. It has been reported that  $\text{Ba}(\text{OH})_2$  does not completely hydrolyze phospholipids, and many investigators prefer the hydrolytic agent introduced by Thannhauser, namely, methanol saturated with  $\text{HCl}$  gas (1, 2, 11). The results shown in Tables III to V indicate, however, that, as used here,  $\text{Ba}(\text{OH})_2$  does hydrolyze phospholipids completely. If this were not so, it is extremely unlikely that ratios for choline to phosphorus close to unity could be so regularly obtained for plasma of two species by two widely differing extraction methods (Tables III to V).

In at least two other instances the absence of  $\text{N C C}$  phospholipids in plasma has been observed. Artom reported that after the feeding of oil to rabbits the level of  $\text{N C C}$  phospholipids in the plasma frequently dropped to zero (11). Williams *et al.* reported the complete absence of cephalin from the plasma of two dogs suffering from propyl disulfide anemia (12).

In view of the present findings it seems reasonable to conclude that the transport of fatty acids by way of plasma phospholipids involves only those phospholipids that contain choline.

#### SUMMARY

1 A study was made of the choline and phosphorus contents of plasma phospholipids of man and dog in the postabsorptive state.

2 Judging from the molar ratios of choline to phosphorus, practically all plasma phospholipids are of the choline-containing type. 5 per cent or less of the plasma phospholipids of man and dog was found to contain no choline.

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# A NUTRITIONAL STUDY OF HUMAN GLOBIN IN RATS

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Human blood cells are obtained in large quantities from the processing of human plasma. Various uses for these cells have been investigated. Red blood cell transfusions have been helpful (1) in various types of anemia, and a blood cell powder seems promising in healing wounds (2). Enzymatic digests for a bacteriological medium (3) and a purified hemoglobin solution as a blood substitute (4) have been prepared from human red blood cells.

Globin and globin hydrolysates are possible products from the human red blood cells which might be used to supply homologous protein for human parenteral nutrition. For such products it is essential that all of the indispensable amino acids (5) be present in adequate amounts. The available analytical data for hemoglobin (6-12), the most abundant protein in these cells, indicated that it was nutritionally complete. However, some of the investigations are of hemoglobin or globin from a species other than man and even though there is a similarity of function of all of the hemoglobins, their composition does vary to some extent (8, 11). Moreover, the analytical procedures for certain amino acids are not accurate and the data obtained some years ago are difficult to assess. Accordingly nutritional experiments and additional analytical data were considered desirable. Recently, since our experiments were started, a complete analysis of the essential amino acids in hemoglobin has been reported (13) as the average of various species, including the human,<sup>1</sup> which differ very little. The deficiency in isoleucine revealed by this analysis is confirmed by the present nutritional experiments and analytical data.

## *Methods and Material*

The preparation of the globin was based on the method of Anson and Mirsky (14). The detail of the procedure employed was as follows:

1 liter of packed human blood cells was laked in 2 liters of distilled water and 170 cc of cold 2 N hydrochloric acid were added slowly. After approximately 5 minutes, the resulting hematin-globin hydrochloride solution was stirred into 24 liters of acetone containing 180 cc of 2 N hydrochloric acid. The precipitate was filtered off, evenly suspended in 6 liters of acetone, and then filtered again. The washing procedure was repeated until

<sup>1</sup> Personal communication to C. A. Z.

human globin We concluded that since our preparations had been dried in an oven at 55–85° for approximately 18 hours the availability of the lysine had been impaired as has been observed for casein that had been heated to higher temperatures (19) A group of rats fed a diet containing 18 per cent of globin that had been dried at room temperature supplemented only

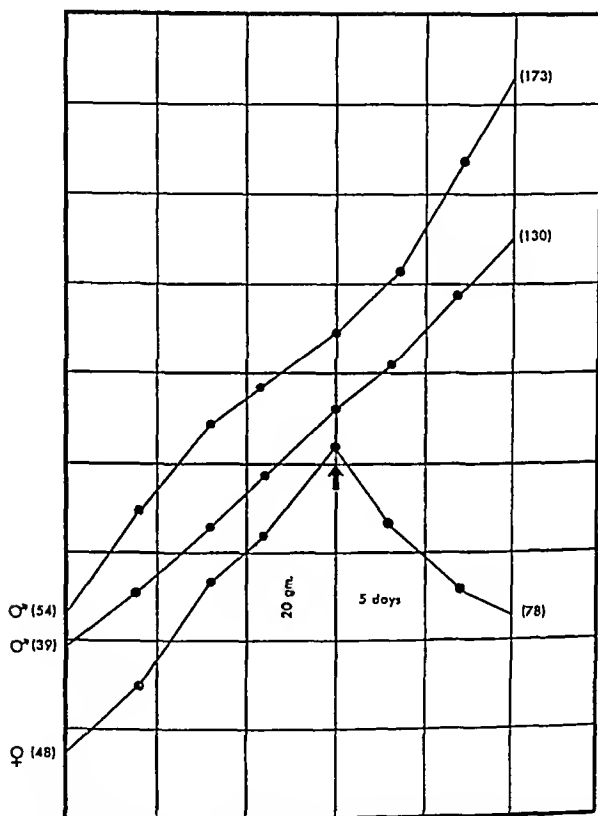


FIG 1 Growth of rats on an 18 per cent casein diet Initial and final weights are given in parentheses On the lowest curve the arrow denotes replacement with a globin diet

with 18.4 gm of *dl*-isoleucine per kilo of diet grew as shown in Table I and Fig 3 A comparison of these animals with the casein controls shows that the principal deficiency was overcome with isoleucine alone However, a comparison of this group with that on the lysine-isoleucine diet, with due consideration to the sex ratio in each group, suggested that the

availability of the lysine in human globin may be impaired slightly even by drying at room temperature. Further investigation of this point is desirable.

### DISCUSSION

These experiments show that the principal nutritional deficiency in human globin is a lack of isoleucine. This deficiency has been confirmed by the analytical data of Block (13) and microbiological assays by the method of Shankman, Dunn, and Rubin (20).<sup>6</sup>

Our observations suggest that the lysine in human globin may be especially sensitive to drying. The effect of heat on the lysine in casein has been given considerable study (19, 21, 22). In heated casein, although the lysine

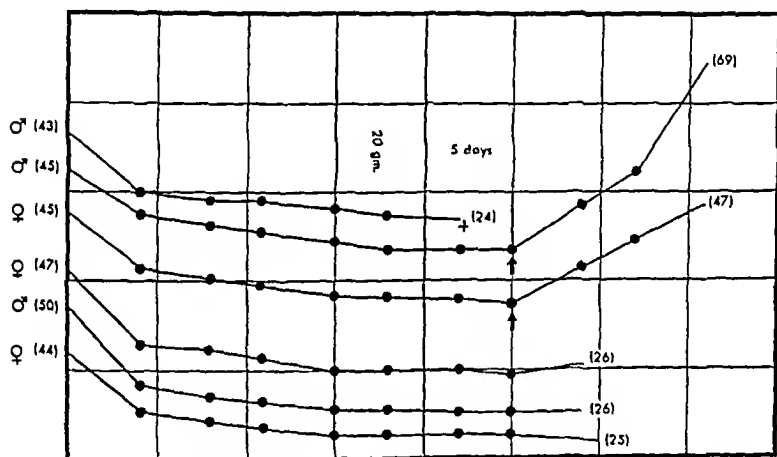


FIG 2 Growth of rats on 18 per cent (upper three curves) and on 23 per cent (lower three curves) globin diets. The cross signifies death, the arrows denote replacement with an 18 per cent casein diet. Initial and final weights are given in parentheses.

is not available nutritionally (19, 22), analytical methods (21) have shown that it is still present. Studies in this laboratory have shown that the lysine content of human globin is not reduced by drying (18).

Whipple and his coworkers in a recent (23) as well as in earlier papers stated that all of their attempts to regenerate plasma protein in dogs depleted of plasma with globin or hemoglobin were futile. In several instances (24, 25), they reported that the proteins of red blood cells were

<sup>6</sup> A hydrolysate of human globin analyzed by microbiological assay contained 17 per cent leucine, 0.29 per cent isoleucine, and 12.2 per cent valine (calculated to 17.0 per cent nitrogen). These data are in fair agreement with the 16.6, 1.5, and 8.2 per cent respectively reported by Block and Bolling (13).

and 250 cc Florence flasks are half filled with the medium and autoclaved at 10 pounds pressure for 45 and 15 minutes respectively. After the medium is autoclaved, 50 per cent sterile glucose solution is added to the large flasks to give a concentration of 2 per cent of glucose. Glucose need not be added to the small flasks which are seeded from slants and used as starters. All flasks are incubated for 24 hours at 25°. The organisms in the large flasks are collected in a Sharples centrifuge and dried with acetone. The yield is about 0.70 gm per liter of medium.

The usual Warburg equipment is employed under the following conditions to determine the activity of the acetone-dried powder and to perform the subsequent analyses. In the flasks are placed 2.0 cc of 0.2 M phosphate buffer, pH 6.0, 0.5 cc of 0.067 M *L*-lysine (9), and 2.5 mg of dried organisms.

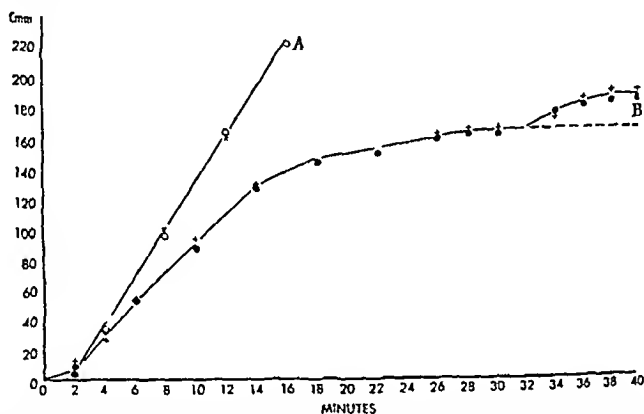


FIG. 1. Duplicate results of the liberation of gas by 2.5 mg of *Bacterium cadaveris* powder from 0.5 cc of 0.067 M *L*-lysine (Curve A) and 0.5 cc of 0.0163 M *L*-lysine (Curve B). At 32 minutes on Curve B, 0.25 cc of 2 N HCl was introduced.

suspended in 0.5 cc of H<sub>2</sub>O. The flasks are shaken at 100 oscillations per minute in a bath at a temperature of 30°. The activity,  $Q_{CO_2}$  (cmm of CO<sub>2</sub> per mg per hour), is calculated from the evolution of CO<sub>2</sub> during the first 12 minutes.

The  $Q_{CO_2}$  of the acetone dried powder is 300 to 600. Gale and Epps (9) obtained a  $Q_{CO_2}$  of 860 per mg of carbon, or roughly 1700 per mg of solid. The use of tryptic digests of casein instead of casamino acids in the medium did not increase the activity of our material. In Fig. 1 is shown the liberation of CO<sub>2</sub> from 0.5 cc portions of 0.067 M and 0.0163 M *L*-lysine. The curve for the former solution represents a determination of the activity of the enzyme. The curve obtained with the 0.0163 M solution exemplifies the liberation of free and bound CO<sub>2</sub>. The latter is obtained by adding

0.25 cc of 2 N HCl after the action of the enzyme on the substrate is complete.<sup>2</sup> The total  $\text{CO}_2$  is corrected for 5 c mm contributed by the enzyme solution. In Fig. 2 the results obtained with various dilutions of 0.0183 M l-lysine are presented.

The experiments with protein hydrolysates were performed in the same manner as with lysine. The  $\text{CO}_2$  was liberated in the same period of time as from the solutions of pure lysine. Corrections were made for  $\text{CO}_2$  in the hydrolysate (the gas liberated by the addition of 0.25 to 0.50 cc of 2 N HCl) which in most cases was negligible and never exceeded 5 c mm. The salt in the hydrolysates was kept at a minimum, since we had observed that suspending the enzyme in 10 per cent NaCl caused a reduction in

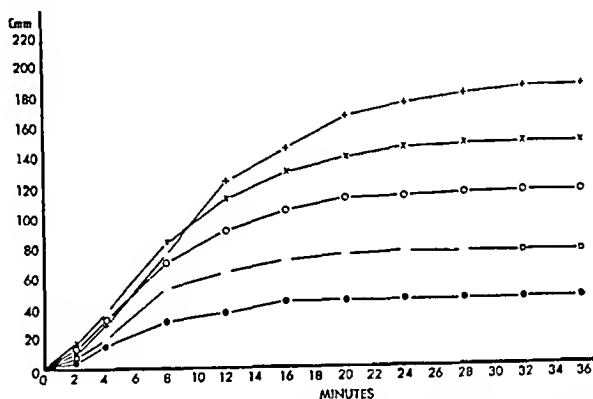


FIG. 2 The liberation of gas by 2.5 mg. of *Bacterium cadaveris* powder from 0.0183 M l-lysine. For the top curve 0.5 cc of the lysine solution was used, for the others, in descending order, the equivalents of 0.4, 0.3, 0.2, and 0.1 cc were used.

activity which increased with time. Each hydrolysate was also tested with a known amount of lysine added as a check on the activity of the enzyme.

<sup>2</sup> The bound  $\text{CO}_2$ , when not measured directly, was calculated from the free  $\text{CO}_2$  with the factor 0.128. This is the average factor for numerous experiments with various amounts of  $\text{CO}_2$  liberated. It was hoped to avoid this correction by carrying out the reaction at a lower pH. Gale and Epps (9) performed their experiments, some of which were with the purified enzyme, at pH 6.0. Gale (10) has reported that the optimum activity of decarboxylases obtained in a cell-free condition from various organisms is at a higher pH than the activity in the intact cells, which were most active between pH 2.5 and 5.5. Accordingly we hoped to have greater activity, since we were using intact cells, and less  $\text{CO}_2$  bound at lower pH values. However, experiments with this organism at pH 5.0 showed a 21 per cent decrease in activity from pH 6.0. Analytical experiments at pH values at which bound  $\text{CO}_2$  is negligible are not practical for this reason.



in the presence of the protein substrate. Samples of the lysine rich hydrolysates under test were added to the phosphate buffer but hydrolysates of the lysine-poor zein and gliadin were adjusted to pH 6.0 and 2.5 cc portions were used without the addition of phosphate.

The precision of the results obtained with protein hydrolysates is shown by the data obtained with human globin dried at 55° and hydrolyzed with HCl. With an 11.8 mg sample (moisture- and ash-free) in six analyses 1.26, 1.34, 1.33, 1.34, 1.25, and 1.29 mg of lysine were found, with a standard deviation of  $\pm 0.04$ . The lysine content is  $11.0 \pm 0.33$  (s.d.) per cent. In general the results with the other hydrolysates fell within these limits.

The accuracy of the data obtained with pure lysine and with lysine added to protein hydrolysate is shown in Table I.

TABLE I  
*Recovery of Pure Lysine and Lysine Added to Protein Hydrolysates*

Substance and amount	Lysine in protein	Lysine found	Recovery
	mg	mg	per cent
1.19 mg lysine		1.18	99.0
1.34 " "		1.35	100.7
1.07 " "		1.09	102.0
0.80 " "		0.84	105.0
0.27 " "		0.28	103.7
0.73 " " + 59.5 mg gliadin hydrolysate	0.70	1.38	98.5
0.73 mg lysine + 105 mg zein hydrolysate	0.06	0.78	99.0
0.61 " " + 6.9 " horse globin hydrolysate	0.70	1.29	98.5

The data for the pure lysine are taken from the curves shown in Figs. 1 and 2.  
\* 1.0 mg of lysine will theoretically liberate 153 c mm of CO.

The following protein hydrolysates were studied:

1. Casein, Pfanzstahl, 2.3 per cent ash, 7.6 per cent volatile matter;<sup>3</sup> hydrolyzed with 8 N H<sub>2</sub>SO<sub>4</sub> for 15 to 20 hours, the sulfate was removed with Ba(OH)<sub>2</sub>. The protein equivalent in the hydrolysate filtrate was calculated by multiplying the N content by 6.55.<sup>4</sup>

2. Casein, same as (1), hydrolyzed with 1.1 N HCl for 18 hours, most of the HCl was removed by vacuum distillation and the hydrolysate neutralized and adjusted to volume. The analytical data are corrected for ash and volatile matter.

<sup>3</sup> The volatile matter was determined by heating the proteins for 1 hour at 100°.

<sup>4</sup> This was calculated after the removal of BaSO<sub>4</sub> and therefore the accuracy of the data will depend on the limitations of such a procedure.

3 Casein heated at 150° for 65 minutes, hydrolyzed the same as for (2) above

4 Gliadin, Pfanstiehl, 1.65 per cent ash, 8.7 per cent volatile matter. The hydrolysate was prepared as for (1) above. The protein equivalent in the hydrolysate filtrate was calculated with the factor 5.70.

5 Zein, Pfanstiehl, 0.60 per cent ash, 6.6 per cent volatile matter. The hydrolysate was prepared as for (1) above. The protein equivalent in the hydrolysate filtrate was calculated with the factor 6.50.

6 Human globin, prepared essentially by the method of Anson and Mirsky (11). The free globin was prepared from the hydrochloride by taking that portion which precipitated when the hydrochloride was neutralized at room temperature, dried at 55°, 1.3 per cent ash, 11.1 per cent volatile matter, hydrolyzed as for (1). The protein equivalent of the hydrolysate filtrate was calculated by multiplying the N content by 5.70.

7 Human globin, same as (6) but hydrolyzed with 1.1 HCl, hydrolysate treated as for (2).

8 Human globin, prepared as in (6), dried at 100°, 0.3 per cent ash, 2.2 per cent volatile matter. The hydrolysate was prepared as for (2).

9 Horse globin, prepared as in (6), 1.1 per cent ash, 12.1 per cent volatile matter, hydrolysate prepared as for (2).

10 Soybean fluff flour, Glidden, 5.0 per cent ash, 5.3 per cent volatile matter, 53.5 per cent protein, 7.90 per cent N. The hydrolysate was prepared as for (2).

The analyses of these hydrolysates are summarized in Table II.

#### DISCUSSION

The content of lysine found in casein is higher than the 5.9 per cent reported by Block and Bolling (3), and the 6.3 per cent (7.8 per cent lysine N) obtained by Tristram (6) with the same method. However, our value agrees closely with the 8.1 per cent (10.22 per cent lysine N) reported by Albanese for his electrolytic method (7) and the tentative 7.6 per cent reported by Dunn (12)<sup>5</sup> for a microbiological method.

The nutritional quality of casein, impaired by heating at 150° for 65 minutes, can be restored by adding lysine (18). However, Block *et al.* (19) have found that the lysine could be isolated quantitatively from casein dried in this manner. Our analytical data are essentially confirmatory. These findings suggest strongly that in heated casein lysine is in a combination which can be split by acids but not by the intestinal enzymes. The temperature of drying likewise had no influence on the lysine content of human globin.

<sup>5</sup> We have learned recently from Professor Dunn that subsequent research, soon to be published, has given the value 8.3 per cent.

The lysine found in gliadin is higher than the 0.64 per cent obtained by Osborne *et al* (13) by the direct method of Kossel. However, it agrees with

TABLE II

*Comparison of Lysine Content of Various Proteins by Decarboxylase Method and Other Methods*

Protein	Method	Size of sample	Lysine
		mg	per cent
Casein	H <sub>2</sub> SO <sub>4</sub> hydrolysis, decarboxylase	10.5-16.5	8.1*
	HCl hydrolysis, decarboxylase	8.0-13.0	7.5
	Dried at 150° for 65 min, HCl hydrolysis, decarboxylase	10.8-14.4	7.0
	Electrolytic (7)		8.1
	Microbiological (12)†		8.3
	Block (3)		5.9
	" (6)		6.3
Gliadin	H <sub>2</sub> SO <sub>4</sub> hydrolysis, decarboxylase	60-80	1.17*
	Van Slyke (13)		1.21
	Kossel (13)		0.64
Zein	H <sub>2</sub> SO <sub>4</sub> hydrolysis, decarboxylase	105-131	0.06*
Human globin	Dried at 55°, H <sub>2</sub> SO <sub>4</sub> hydrolysis, decarboxylase	7.3-10.0	9.9*
	Dried at 55°, HCl hydrolysis, decarboxylase	10.8-16.0	11.0
	Dried at 100°, HCl hydrolysis, decarboxylase	8.1-13.5	10.1
	Block (14)‡		8.0
	HCl hydrolysis, decarboxylase	14.3-17.9	10.1
Horse "	Electrolytic (7)		10.3
	Van Slyke (15)		10.2
	Vickery (5)		8.1
	Block (16)		8.1
	HCl hydrolysis, decarboxylase	16.0-24.0	3.32
Soya fluff flour			6.7§
Soy bean meal	Block (17)		5.4§

\* Since the size of these samples was calculated from the N content of the hydrolysate after the removal of BaSO<sub>4</sub>, the accuracy of these data will depend on the limitations of such a procedure.

† We have learned recently from Professor Dunn that subsequent research, soon to be published, has given the value 8.3 per cent.

‡ Dr. Block has informed us that the hemoglobin data represented the average of various species, including the human, all of which were about the same.

§ Calculated to 16 per cent N.

the 1.21 per cent obtained by the same authors with the Van Slyke method, as is the case for the globin data. The amount of lysine in zein is very small and probably is contributed by contaminating proteins. Zein, with

a molecular weight of 35,000 (17), would, if it contained 1 molecule of lysine, have a content of 0.42 per cent. Evidently this is a protein that in the pure form contains no lysine.

Human globin and horse globin contain about the same amount of lysine. Other reports show that the hemoglobins of various species contain about the same amount of lysine (5, 14, 16<sup>6</sup>). However, our data are considerably higher than the 7.7 to 8.1 per cent reported in these papers. It is of interest that our data are in close agreement with the 10.3 per cent lysine (11.30 per cent lysine N<sup>7</sup>) obtained by Albanese with his electrolytic method (7) for horse hemoglobin. Data obtained by the Van Slyke method are of the same magnitude, Van Slyke (4) reported 10.0 per cent (10.9 per cent lysine N) in ox hemoglobin, Hunter and Boisook (15) 10.2 per cent (11.1 per cent lysine N) in horse globin.

The lysine in the soy bean flour when calculated to 16 per cent N becomes 6.72 per cent. Block and Bolling (14), reported 5.4 per cent in a commercial soy bean meal. Some of the differences in the lysine analyses reported for various proteins may be due to the methods of preparation of the proteins (7). However, in general the data obtained with Block's method (3, 5, 6, 14, 16) are about 20 per cent lower than the results obtained by us as well as by others (4, 7, 12, 15).

Gale and Epps (9) have found *Bacterium cadaveris* specific in its decarboxylase action. We have tested it against arginine, histidine, and glutamic acid, the amino acids most likely to be acted on (20), and found it unreactive. The small amount of CO<sub>2</sub> liberated from a zein hydrolysate (the enzyme liberated CO<sub>2</sub> quantitatively from lysine added to this hydrolysate) indicated that other amino acids as well are not acted on by it. This specificity suggests that the lysine content found for the proteins analyzed represents an approximation to their true values. This is borne out by the similar values found by others. With more complex substrates this method should be applied with caution, since active enzymes other than the decarboxylase may be present. However, the results with the carbohydrate-containing soy bean flour are of the expected magnitude in comparison with Block and Bolling's result (14).

Lysine analyses can be very easily performed in a short time by the use of this method. The amount of sample required is small, for a protein containing 10 per cent lysine 100 to 200 mg are sufficient. Hydrolysis with HCl is more convenient than with H<sub>2</sub>SO<sub>4</sub>, since manipulation of the

<sup>6</sup> Dr. Block has informed us that the hemoglobin data represented the average of various species, including the human, all of which were about the same.

<sup>7</sup> The lysine N here and elsewhere is the per cent of total N. For calculating the per cent lysine in globin or hemoglobin, protein N is converted to protein with the factor 5.70. No correction is made for hematin N, which is about 2 per cent of the total N.



# ELECTROPHORETIC AND ULTRACENTRIFUGAL ANALYSES OF GLOBIN COMPONENTS

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In a previous communication (1) we reported the presence of two electrophoretic components in solutions of denatured as well as native cattle globin prepared according to Anson and Mirsky's method (2) and in globin preparations prepared by fractionation with ammonium sulfate according to the method of Roche and Combette (3). The relative proportions of the components in these preparations were about the same, that is, about 60 per cent of a "fast" component and 40 per cent of a "slow" component, when examined between pH 2.5 and 3.6. Similar results were also reported by Munro and Munro using cattle and human globins (4). The titration curves between pH 5.5 and 6.8 and the sulfur content of the two components isolated by electrophoretic separation were different.

Extension of previous experiments showed that the two electrophoretic components were also present in globins prepared from human and crystalline horse hemoglobin. Moreover, they could be demonstrated in acidified solutions of hemoglobin in which the protein was not exposed to denaturing organic solvents used to separate it from the iron porphyrin complex in the preparation of globin. It was of further interest to examine the electrophoretic behavior of the globin components at different hydrogen ion concentrations and to investigate their sedimentation rates in the ultracentrifuge.

## EXPERIMENTAL

Most of the electrophoresis experiments were carried out in the standard Tiselius cell (capacity, 11 ml). Several separatory experiments were made, however, in a macro cell having a capacity of 100 ml. This cell consisted of two tall sections and was operated with compensation so that portions of pure fast and slow fractions could easily be isolated in the upper channels.

Sedimentation constants were determined in an air-driven vacuum ultracentrifuge (5), the sedimenting boundaries being recorded by the Longworth scanning method (6). The analyses were made at room temperature, which varied from 17–23°. Since the temperature of the rotor spinning at 48,000 R.P.M. increased about 1.2° per hour, it was possible to estimate the average temperature over any desired time interval during a run. All values of

sedimentation constants were reduced to  $20^\circ$  and pure water. In the ultracentrifuge patterns the white reference lines on each side of the pattern were made by light-transmitting holes in the balance cell, the one on the left being 5.706 cm from the center of rotation. A rotor speed of 48,000 R P M, giving a field of  $170,000 \times g$  at the center of the cell, was used in most of these experiments.

Diffusion constants were determined in a Tiselius electrophoresis cell used as a diffusion cell (7) at a temperature of  $1^\circ$ , the values thus obtained being corrected to  $20^\circ$ . During each experiment three or four scanning photographs of the boundaries were taken and the diffusion constants calculated from the width of the pattern at the inflection point of the curve (8). These values, which varied in some experiments as much as 12 per cent, were averaged and are recorded in Table III.

### *Materials and Results*

*Electrophoresis of Horse Globin*—Horse globin was prepared in the usual manner from a thrice recrystallized hemoglobin solution. The hemoglobin was converted into its carbon monoxide compound, cooled to  $1^\circ$ , and mixed with an equal volume of 0.1 N hydrochloric acid. It was allowed to stand for  $\frac{1}{2}$  hour during which time the solution turned a dark brown, indicating splitting of the acid hematin from the globin. The globin was precipitated by pouring it into cold acetone containing 0.1 mole of HCl per liter, quickly filtered through a Buchner funnel, and washed first with cold acetone and then with cold ether, and air-dried. A 1 per cent solution of this globin was prepared in glycine buffer ( $\mu = 0.10$ , pH 2.5) and in sodium acetate buffer (pH 4.0). The solutions were dialyzed against the buffer and then subjected to electrophoresis. As can be seen from Fig. 1, two components were present at both pH values and the patterns obtained at pH 2.5 were similar to those obtained previously with cattle globin.

For the sake of comparison, samples of carbon monoxide hemoglobin prepared from thrice recrystallized horse oxyhemoglobin were analyzed electrophoretically. As is seen in Fig. 2, evidence of splitting was obtained at pH 6.8 in phosphate, at pH 4.0 in acetate, and at pH 3.0 in glycine buffer ( $\mu = 0.10$ ). The pattern obtained at pH 4.0 resembles that obtained at the same pH with horse globin except that there is a trace of a third component of low mobility in the hemoglobin sample. This third slow moving component is also evident in the descending pattern at pH 3.0. The patterns obtained for hemoglobin near the isoelectric point were taken after 17 hours of electrophoresis, but are similar to those of cattle globin at pH 7.1 (cf Fig. 4). Electrophoresis of horse serum albumin for similar lengths of time at pH 7.4 and 4.8 failed to show any evidence of more than one component.

*Electrophoresis of Human Globin*—Human globin was prepared in a manner similar to that used for the preparation of horse globin except that the hemoglobin was not crystallized. Electrophoresis was carried out at pH 2.5 in glycine buffer, and at pH 4.0 in sodium acetate buffer (Fig 1). At

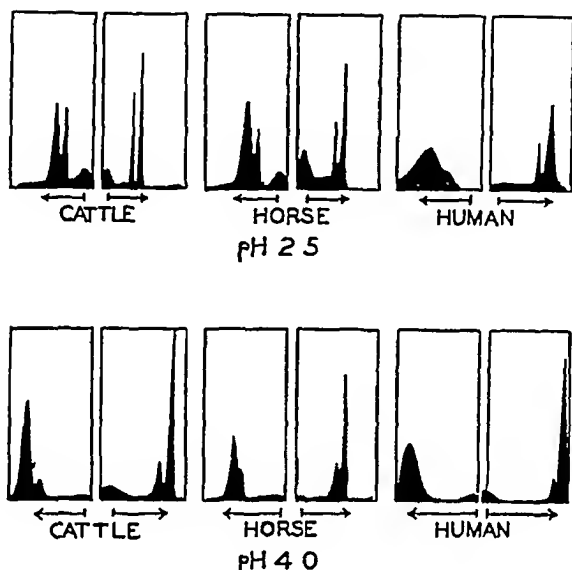


FIG 1 Electrophoresis patterns of cattle, horse, and human globin. Left, descending, right, ascending

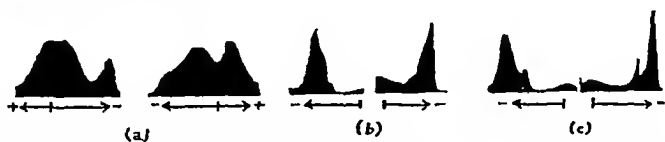


FIG 2 Electrophoresis patterns of horse hemoglobin (a) at pH 6.8, in 0.02 M sodium phosphate buffer + 0.05 M NaCl, after 17 hours at 6.5 volts per cm, (b) at pH 4.0, in 0.15 M sodium acetate buffer + 0.076 M NaCl after 2 hours, at 5.7 volts per cm, (c) at pH 3.0, in 0.1 M glycine + HCl buffer + 0.08 M NaCl, after 1 hour and 45 minutes, at 5.3 volts per cm

pH 2.5, two fractions appeared as in the other species, but boundary spreading on the descending side was appreciably greater. At pH 4.0 the two fractions were not resolved on the descending side.

Similar experiments were also carried out with human hemoglobin. This was prepared by washing fresh red blood cells five times with equal volumes



of 0.85 per cent NaCl solution and laking with distilled water. Stromata were removed by ultracentrifugation at about  $100,000 \times g$  for 20 minutes. Analyses were made at pH 4.0 in sodium acetate buffer and at pH 2.5 in glycine-hydrochloric acid buffer. At the higher pH value two distinct compo-



FIG 3 Electrophoresis pattern of acidified human hemoglobin at pH 2.5, 0.1 M glycine + HCl buffer + 0.08 M NaCl after 2 hours, at 4.4 volts per cm

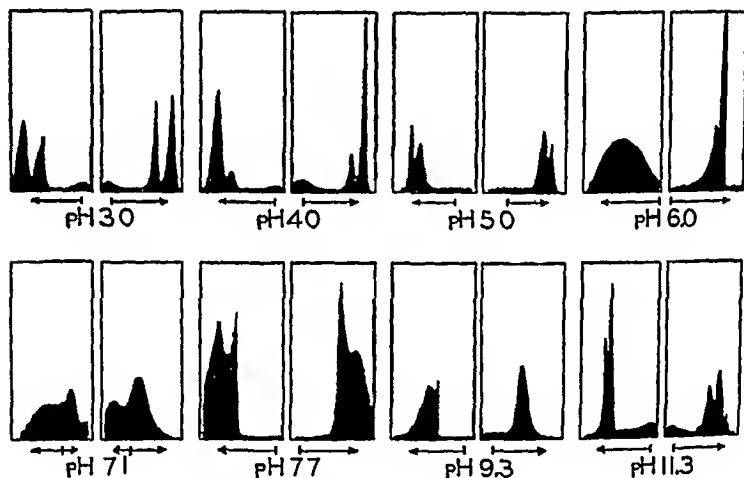


FIG 4 Electrophoresis patterns of cattle globin at various pH values. The buffers used are given in Table I. The patterns were obtained after a field of about 5 volts per cm was applied for the following lengths of time: pH 3.0, 2 hours; pH 4.0, 3 hours; pH 5.0, 4 hours; pH 6.0, 7 hours; pH 7.1, 20 hours; pH 7.7, 15 hours; pH 9.3, 4 hours; pH 11.3, 3.25 hours. Patterns with arrows pointing left are of the descending limb, right, ascending limb, except at pH 7.1, where components migrate in both directions, arrows pointing together indicate cathodic component, away from each other, anodic.

nents were not evident after 6.5 volts per cm had been applied for 7 hours, but both ascending and descending boundaries were much broadened. At pH 2.5, two fractions appeared (Fig 3), the pattern being almost identical to that of human globin in the same buffer (Fig 1).

*Effect of pH on Electrophoretic Pattern of Globin*—"Native" cattle globin

was subjected to electrophoresis at various pH values ranging from pH 2.5 to 11.3 in buffers having an ionic strength of 0.10. The patterns and mobilities obtained are given in Fig. 4 and Table I respectively.

It was previously reported (9) that no appreciable separation of components takes place between pH 6.0 and 9.0. These results were obtained after a relatively short period of electrophoresis. If, however, electrophoresis was carried out for a sufficient length of time (10 hours), patterns indicating two components were obtained at all pH values. At pH 7.1 fractions moving in opposite directions were found. Exact analysis and determination of the isoelectric points of the separated fractions have not yet been

TABLE I  
Effect of pH on Mobility of Cattle Globin

Concentration, 1 per cent in a buffer of  $\mu = 0.10$

pH	Buffer	Mobilities $\times 10^4$ sq. cm. per sec. volt			
		Descending		Ascending	
		Fast	Slow	Fast	Slow
2.5	G <sub>1</sub>	8.7	5.9	9.4	7.3
3.0	"	8.6	6.3	9.0	7.1
4.0	A	5.5	4.3	6.8	4.8
5.0	"	3.9	3.2	4.1	3.5
6.0	P	2.4	1.9	2.5	2.3
7.1	"	0.32	-0.02	0.20	-0.13
7.7	"	-1.3	-1.0	-1.6	-1.2
9.3	G <sub>2</sub>	-3.1	-2.7	-2.7	-1.2
11.3	"	-7.0	-3.6	-7.6	-5.2

\* G<sub>1</sub> = 0.10 M glycine + HCl + NaCl, A = 0.15 M acetate + NaCl, P = 0.02 M phosphate + NaCl, G<sub>2</sub> = 0.10 M glycine + NaOH + NaCl

possible. Carefully separated fractions at low pH values were largely precipitated as the pH approached neutrality.

*Sedimentation of Cattle Globin Fractions*—Solutions of native cattle globin were examined at pH 3.0 in the ultracentrifuge at protein concentrations of 0.5, 1.0, and 2.0 per cent. At the higher concentrations a large portion of polydisperse material was present and an appreciable amount of aggregation took place during centrifugation, as is evident from the patterns of Fig. 5. The first pattern was taken after centrifugation at  $170,000 \times g$  for 1 hour and has an area equivalent to a protein concentration of 1.4 per cent instead of 2 per cent, the original concentration of the solution, furthermore, the area of the two components in the last pattern corresponds to a concentration of a little more than 0.5 per cent, the major portion of the material, therefore, had not sedimented as individual com-

ponents but had continuously formed heavy aggregates during centrifugation. The sedimentation constants of the two discernible components



FIG 5 Ultracentrifuge patterns of native cattle globin at pH 3.0. Patterns taken at 1 hour intervals, rotor speed, 48,000 R.P.M., protein concentration, 2.0 per cent

TABLE II

*Sedimentation Constants of Globin, Hemoglobin, and Electrophoretic Fractions Obtained from Them*

	Preparation	Concentration	pH	Buffer*	$\mu$	S <sub>20</sub> †
		per cent				
Native cattle globin	Unseparated	0.5	3.0	G <sub>1</sub>	0.10	0.9
	"	1.0	3.0	"	0.25	0.9
	"	2.0	3.0	"	0.10	1.0
						3.3
	Fast	0.5	3.0	"	0.10	3.6
Fractions obtained from horse Hb	Slow	0.25	3.0	"	0.10	1.6
	"	0.12	5.0	A	0.10	1.8
	Anodic	0.50	7.1	P	0.20	4.8
	Unseparated	0.85	7.1	"	0.20	4.4
	Cathodic	0.30	6.8	"	0.20	5.3
	Anodic	0.80	6.8	"	0.20	4.9
	Slow	0.30	3.0	G <sub>1</sub>	0.10	1.9
Fractions obtained from human Hb	Unseparated	0.8	3.0	"	0.10	2.7
	"	0.5	4.0	A	0.10	3.6
	"	0.1	4.0	"	0.10	2.3
	Fast	0.1	4.0	"	0.10	2.6
	Slow	0.1	4.0	"	0.10	2.0

\* G<sub>1</sub>, P, and A indicate buffers as in Table I

† S<sub>20</sub> = sedimentation constant in units of 10<sup>-13</sup> reduced to water at 20°

(Table II) were 1.0 and 3.3 Svedberg units, the concentration of the lighter fraction apparently decreased more rapidly during centrifugation than that of the heavier

The same phenomenon was observed when the original protein concentration was 0.5 per cent. By the time the first pattern was recorded (Fig 6), the area corresponded to a concentration of only 0.2 per cent, but the decrease thereafter was not appreciable.

Electrophoretically separated fast and slow fractions had sedimentation constants of 3.6 and 1.6 Svedberg units at pH 3.0. Unlike the unseparated material there was no noticeable decrease in concentration during centrifugation (Figs 7 and 8) and both fractions appeared monodisperse, though small amounts of heavier or lighter particles may have been present.

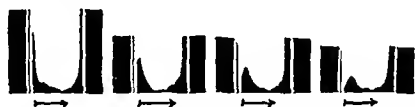


FIG 6 Ultracentrifuge patterns of native cattle globin at pH 3.0. Patterns taken at 1 hour intervals, rotor speed, 48,000 r.p.m., protein concentration, 0.5 per cent.



FIG 7 Ultracentrifuge patterns of the fast cattle globin fraction at pH 3.0. Patterns taken at 1 hour intervals, rotor speed, 48,000 r.p.m.



FIG 8 Ultracentrifuge patterns of the slow cattle globin fraction at pH 3.0. Patterns taken at 1 hour intervals, rotor speed, 45,000 r.p.m.

Another slow fraction which was separated at pH 5.0 had a sedimentation constant of 1.8 Svedberg units.

A sample of human globin examined at pH 7.4 in a solution having an ionic strength of 0.20 appeared to be homogeneous and had an  $S_{20} = 5.3$  Svedberg units.

*Sedimentation of Hemoglobin Fractions*—After electrophoresis of horse carbon monoxide hemoglobin for 18 hours at 5.7 volts per cm. in a 0.02 M Na phosphate buffer containing 0.05 M NaCl at pH 6.8 (Fig 2, a) small samples of anodic and cathodic fractions were taken for analysis in the ultracentrifuge.  $S_{20}$  for the cathodic fraction was found to be 5.3 Svedberg units and

for the anodic fraction 4.9 units. Both values were slightly higher than that found for the same hemoglobin before electrophoresis (4.4 Svedberg units). Analysis of a sample of the electrophoretic slow fraction separated from horse hemoglobin at pH 3.0 gave  $S_{20} = 1.9$  Svedberg units.

Samples of human hemoglobin were studied at concentrations of 0.5 and 0.1 per cent at pH 4.0 and of 0.8 per cent at pH 3.0. At pH 4.0 the sedimentation constant was found to vary greatly with concentration, indicating further dissociation as the concentration was reduced. The pres-



FIG. 9. Ultracentrifuge pattern of human hemoglobin at pH 4.0. Patterns taken at 1 hour intervals, rotor speed 48,000 R.P.M.

TABLE III  
Molecular Weights of Globin Preparations

Sample	pH	Buffer	$\mu$	$S_{20}^\dagger$	$D_{20}^\ddagger$	$M_0$
Human, native, No. 226B	7.4	P	0.20	5.3	6.7	76,000
Cattle, " "	7.4	"	0.20	5.0	5.8	85,000
55-65% ammonium sulfate ppt	7.4	"	0.20	3.4	6.7	50,000
Fast, cattle, native	3.0	G	0.10	3.6	7.7	45,000
Slow, " "	3.0	"	0.10	1.8	11.0	16,000

\* P = 0.02 M phosphate + 0.15 M NaCl, G = 0.08 M glycine + 0.03 M NaCl + 0.02 M HCl.

†  $S_{20}$  = sedimentation constant in units of  $10^{-13}$  reduced to water at 20°.

‡  $D_{20}$  = diffusion constant in units of  $10^{-7}$  reduced to water at 20°.

§ The partial specific volume of the globin was assumed to be 0.750.

ence of polydisperse heavy material was indicated by decreasing pattern areas during centrifugation and by extended area of refractive gradient from the bottom of the cell (see Fig. 9). The patterns for an 0.8 per cent solution of hemoglobin at pH 3.0 revealed a single boundary but its area decreased during centrifugation in a similar manner to that of globin and a large gradient extended from the bottom of the cell. This indicated that denaturation resulting from extracting solvent was not responsible for the effects observed with globin.

Although the two electrophoretic fractions of human hemoglobin were not

distinctly separated at pH 4.0, 1 ml samples each were taken from the forward portion of the ascending and the lagging portions of the descending boundaries, both of which showed unusual spreading. Sedimentation constants of these fractions were 2.6 Svedberg units for the fast and 2.0 units for the slow fraction. The patterns showed a single boundary but also indicated the formation of aggregates during centrifugation.

*Molecular Weights*—Because of their polydisperse nature most of the materials studied did not lend themselves to accurate determinations of molecular weight. Diffusion constant determinations were made, however, on a few samples and the molecular weights calculated from these and the corresponding sedimentation constants. These values are recorded in Table III.

#### DISCUSSION

From the foregoing experiments it is evident that the protein of hemoglobin, unlike most other proteins, is extremely unstable even under the most gentle methods of handling. It would appear that the molecule is composed of several (probably four) parts which dissociate with great ease and reassociate to form either smaller, the same, or larger particles than the original one.

It was found that the relative proportion of the electrophoretic fractions in globin solutions changed as the hydrogen ion concentration was altered. If the components were separated by electrophoresis at an acid pH value, at least one of them, the fast fraction, often proved to contain two components on electrophoretic reexamination. The appearance of two electrophoretic fractions with different physical and chemical properties would seem to indicate that not all the parts are identical although the alteration resulting from dissociation is not known.

Although most methods used for the preparation of globin, including that used in our experiments, yield some denatured globin which is insoluble at its isoelectric point, regeneration of part of the globin has been reported, yielding a protein soluble at pH 7.0 and capable of combining with heme to form a complex having identical absorption spectrum (2) and molecular weight (10) as the original hemoglobin. However, it is still questionable whether or not the so called regenerated globin is identical in structure and physical state with the protein moiety of hemoglobin which alone, in our opinion, is entitled to the designation native globin. That globin at neutral pH is polydisperse in the ultracentrifuge has been known (10). Nevertheless, it appears to be significant that the protein obtained from hemoglobin shows two distinct electrophoretic components and that one of the components has a low molecular weight (about 16,000).

## SUMMARY

Two electrophoretic components were found in globins prepared from cattle, human, and horse hemoglobins

The presence of these components was also demonstrated in acidified ( $\text{pH} \leq 4.0$ ) hemoglobin solutions of the same species, the patterns being similar to those obtained with the respective globin preparations

The relative proportions of the fast and slow moving fractions changed as the pH was varied, suggesting that the composition of the fractions was also altered. Upon prolonged electrophoresis, splitting of the boundary could be demonstrated at any pH, and in the region pH 7 both anodic and cathodic migration of globin as well as of hemoglobin took place

Ultracentrifugal analysis of globin solutions at pH 3.0 showed the presence of at least two fractions sedimenting at different rates. The sedimentation constants were approximately 1.6 Svedberg units for the light and 3.6 units for the heavy fraction, however, aggregation to much larger particles took place during centrifugation

Electrophoretically separated fractions showed only one sedimenting component. The fast fraction had a sedimentation constant of about 3.6 Svedberg units and the slow one of 1.8 units

At pH 7.4, globins showed one sedimenting boundary having a sedimentation constant of about 5 Svedberg units. A fraction could, however, be isolated by salting-out with 50 to 65 per cent ammonium sulfate, which apparently had a smaller molecular weight,  $S_{20} = 3.4$  Svedberg units

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# OBSERVATIONS ON THE FIRST STAGES OF CASEIN HYDROLYSIS BY CHYMOTRYPSIN AND TRYPSIN\*

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The fact that the specificity of most proteins is lost when the slightest change is made in the structure of these compounds makes experiments dealing with the first changes which these molecules may undergo during enzymatic hydrolysis of particular interest. This paper presents data on the production of a compound which is representative of the earliest products produced during an enzymatic digestion of a protein.

If 1 mg. of crystalline chymotrypsin dissolved in 1 ml. of water is added to 10 ml. of a solution of 6 per cent casein (pH 7.5) which has been warmed to 50°, the solution will become opaque in about 1 minute. By the use of decreasing amounts of chymotrypsin it can be shown that the rate of this reaction is a function of the concentration of the chymotrypsin. The product formed appears as a milky suspension which cannot be centrifuged down. It dissolves if the digestion tube is plunged into cold water and reappears when the tube is warmed. Since this rennin-like activity of chymotrypsin at pH 7.5 seemed worthy of investigation, the reaction was subjected to quantitative study.

## EXPERIMENTAL

The casein used was "according to Hammersten." Since many fundamental enzyme studies have been made on this mixture, it was not fractionated into its component parts (Linderström-Lang (1)), but was used as purchased. An amount of this casein sufficient for several years of investigation was mixed and stored. Solution of the casein was effected by mixing 12 gm. with 40 ml. of 0.2 N NaOH. Approximately 20 ml. of M/15 phosphate buffer at pH 7.5 plus sufficient water to bring the total volume to 200 ml. were then added.

The chymotrypsin and trypsin were prepared and recrystallized according to the published techniques of Northrop and Kunitz (2). The amount of these materials used is reported in terms of enzyme nitrogen, since our preparations were dried on ammonium sulfate. The amount of ammonium

\* Presented before the American Society of Biological Chemists at Boston (*Federation Proc.*, 1, pt. 2, 117 (1942)).



nitrogen present was determined by analyzing the amount of sulfate ion present in the dried enzyme preparations. Total nitrogen minus ammonium sulfate nitrogen gives the estimate of enzyme nitrogen.

The end-point for the study of the reaction in question was arbitrarily chosen as the amount of turbidity equal to that obtained when 3.6 mg of casein in 3 ml of solution are mixed with 3 ml of 1 per cent gum ghatti and 4 ml of 5 per cent sulfosalicylic acid. This suspension was prepared fresh for each experiment. It is easy to match this end-point during the first few minutes with an accuracy of 10 seconds. As the rate of reaction decreases, matching the end-point becomes slightly more difficult but an accuracy of  $\pm 20$  seconds can be obtained even when the reaction takes 35 minutes.

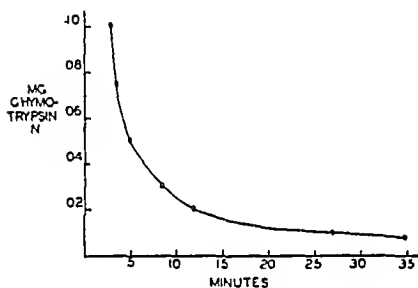


FIG 1

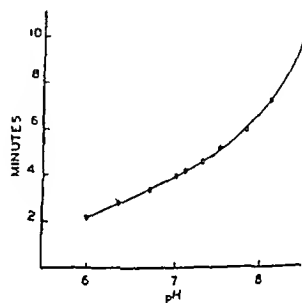


FIG 2

FIG 1 Time required to change 6 per cent casein at pH 7.5 to standard turbidity by varying amounts of chymotrypsin, temperature  $50^{\circ}$

FIG 2 Effect of changing the pH of the substrate (6 per cent casein solutions prepared by using different amounts of dilute sodium hydroxide, the pH ascertained with a Beckman pH meter) on the time required to reach standard turbidity. The enzyme concentration was constant at 0.048 mg of chymotrypsin nitrogen, temperature  $50^{\circ}$

The rate of appearance of this precipitate is not directly related to the amount of amino nitrogen liberated, since this precipitate is more soluble at those hydrogen ion concentrations at which the activity of the protease is greatest. This relationship was studied by using the electrometric formal titration of Borsook and Dubnoff (3) to estimate the amount of amino acid liberated during the time elapsed to complete the reaction.

The experiments were conducted in ordinary glass test-tubes ( $18 \times 150$  mm) suspended in a glass water bath to facilitate visual observations.

Fig 1 presents a curve which shows the time required for different amounts of chymotrypsin dissolved in 1 ml of  $H_2O$  to bring 10 ml of a solution of 6 per cent casein at pH 7.5 to a standard amount of turbidity at a temperature of  $50^{\circ}$ . The relationship between the time of reaction and the concentration of the enzyme is fairly constant. By using this

curve it has been possible to estimate very quickly chymotrypsin concentrations with an error of less than 10 per cent

A trace of calcium is necessary for this reaction, and the amount of calcium adhering to the casein is an important variable affecting the rate of formation of the reaction products. If the casein solution is allowed to stand with 0.5 volume of 20 per cent sodium oxalate for at least 3 hours and the calcium oxalate formed is removed by centrifugation, no precipitate will form as a result of chymotrypsin digestion unless the calcium is replaced.

The rate of appearance of the turbidity is affected by the hydrogen ion concentration of the casein substrate. Fig 2 gives the relationship between pH and the time of appearance of the turbidity. This curve indicates that for the concentrations of enzyme employed (0.048 mg of chymotrypsin nitrogen) the lower the pH the more rapidly the turbidity appears. At pH 9.56 the hydrolytic action of chymotrypsin on casein is approximately as strong as at pH 7.0, yet the turbidity did not appear until after 30 minutes. Since the precipitate appears more readily in weakly acid solution, it is obvious that its appearance is not directly related to the amount of amino nitrogen liberated.

These reactions are not specific for chymotrypsin, since crystalline trypsin has a similar effect on casein substrates, but it takes place at a much slower rate. Even minute amounts of chymotrypsin can be expected to produce the turbidity after sufficient time has elapsed, but when small amounts of trypsin are employed, this turbidity may not appear at all. Trypsin hydrolyzes casein into different segments, so that either less of the insoluble casein product is formed in comparison with chymotrypsin, or else the product is digested more rapidly by trypsin than by chymotrypsin.

Several times as much amino nitrogen are liberated by trypsin before the same turbidity is attained at pH 7.5 and 50°. Table I presents an estimation of the comparative extent of the proteolysis (as determined by the formal titration) brought about by different concentrations of trypsin and chymotrypsin under the condition described above. The third column, which lists the time elapsed to reach the standard turbidity, is the duration of the proteolysis. At these times the reaction was inhibited by adding formaldehyde and titrating electrometrically (3) as rapidly as possible.

It is apparent from the data in Table I that the hydrolysis of the peptide bond is not directly related to the amount of casein product formed or else that very few peptide bonds have to be hydrolyzed to make this product.

The purity of a chymotrypsin or trypsin preparation can be estimated by making use of the information presented in Table I. For example, before the standard end-point was reached, chymotrypsin liberated enough carboxyl groups to give a titration of approximately 0.35 ml of 0.1 N NaOH, whereas enough trypsin must be present to give a titration of approximately

1.25 ml to attain the same end-point. A formol titration between 0.35 and 1.25 ml would indicate that both chymotrypsin and trypsin are present. Examples of the action of mixtures of these enzymes on 6 per cent casein

TABLE I

*Comparison of Chymotrypsin and Trypsin Action on Casein at 50°*

The time required to produce a standard turbidity in a 6 per cent solution of casein at pH 7.5 is given in minutes. The extent of proteolysis which takes place during this time period was estimated by means of a formol titration with 0.1 N sodium hydroxide.

Chymotrypsin	Trypsin	Period	Formol titration
mg N	mg Y	min	ml 0.1 N NaOH
0.0960		2.5	0.40
0.0480		5.0	0.38
0.0288		7.8	0.34
0.0192		11.7	0.35
0.0096		26.0	0.30
	0.1560	6.0	1.30
	0.0780	10.5	1.25
	0.0312	27.8	1.25
	0.0156	50.0	1.21
0.0192	0.0312	6.5	0.55
0.0078	0.0780	9.2	1.20

TABLE II

*Inhibition of Chymotrypsin Digestion of Casein by Hexylresorcinol*

2 mg. of hexylresorcinol were mixed with 0.042 mg. of chymotrypsin nitrogen and permitted to stand for the time designated in the first column. At the end of these time periods, the mixture (contained in 2 ml.) was tipped into 10 ml. of 6 per cent casein solution at pH 7.5 and the time required to produce a standard turbidity at 50° noted in the second column. The third column gives the times to produce the standard turbidity without hexylresorcinol.

Time of contact before mixing with casein	Chymotrypsin and hexylresorcinol	Chymotrypsin and water
min	min	min
0.5	7.3	5.8
15	19.8	5.8
30	24	6.0
60	31	5.8
90	35	6.0
120	45	6.0
180	59	6.0

at pH 7.5, temperature 50°, are given in the last two lines of Table I. This experiment suggests a means of determining the amounts of chymotrypsin and trypsin in an unknown mixture of the two.

Another example of a practical use of the reaction between chymotrypsin and casein is in the study of the effect of denaturants on active proteases. When the time of contact between the denaturant and the protease is an important factor, it is necessary to have a rapid means of estimating the loss of activity of the enzyme. Table II presents the data obtained from an experiment devised to determine the effect of hexylresorcinol on chymotrypsin activity. Hexylresorcinol<sup>1</sup> solutions were prepared by dissolving 100 mg of the crystalline compound in 50 ml of water. 1 ml portions of this solution were mixed with 1 ml of chymotrypsin solution containing 0.042 mg of chymotrypsin nitrogen. After the specified time intervals indicated in the first column of Table II, this mixture was tipped into 10 ml of casein solution which had been brought to 50° in the water bath. Since the room temperature during this experiment was 25°, this was the temperature of the hexylresorcinol-chymotrypsin mixture before it was mixed with the casein. A control determination with water instead of hexylresorcinol solution was performed at each time interval used. The second and third columns give the time elapsed before the standard endpoint was reached for chymotrypsin with hexylresorcinol and for chymotrypsin with water, respectively. The loss of chymotryptic activity brought about by hexylresorcinol is clearly demonstrated by this experiment. Some inhibition takes place even after less than 1 minute's contact of hexylresorcinol with chymotrypsin and the longer the two are together the greater the loss of chymotryptic activity.

A similar experiment shows that heparin will not inhibit this action of chymotrypsin but will strongly inhibit trypsin. This confirms an earlier observation by Horwitt (4). Contrary to expectations, crystalline trypsin inhibitor prepared according to Kunitz and Northrop (5) strongly inhibited this action of both chymotrypsin and trypsin. These inhibitions will be discussed in the following paper in which the methods described here of studying the early stages of casein digestion are further applied.

#### SUMMARY

A fundamental difference between the actions of chymotrypsin and trypsin on casein has been observed and studied.

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<sup>1</sup> This compound was obtained in crystalline form from Sharpe and Dohme, Philadelphia.



# REACTIONS OF TRYPSIN AND CHYMOTRYPSIN WITH HEPARIN, TRYPSIN INHIBITOR, AND HEXYLRESORCINOL

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A study of enzyme inhibitors designed to obtain information on the possible physiological significance of blood antiprotease has produced a number of simple fundamental reactions which have hitherto not been reported. It is the purpose of this paper to record these reactions for the possible benefit of others who may wish to investigate them further.

**Materials**—The trypsin, chymotrypsin, and trypsin inhibitor were prepared in crystalline form according to the techniques described by Northrop and Kunitz (1). The heparin (110 units per mg), rennin, and the crystalline hexylresorcinol were obtained from the manufacturers of these compounds.<sup>1</sup>

## EXPERIMENTAL

Important chemical differences between chymotrypsin and trypsin may be expected from the fact that their isoelectric points are quite different (1). Chymotrypsin has its isoelectric point at pH 5.4, whereas the isoelectric point of trypsin lies between pH 7 and 8. This difference may help partially to explain the following observations which indicate that the precipitation reactions of these two compounds are unlike.

Unless otherwise noted, the concentration of the enzyme and inhibitor solutions used are 1 mg of material per ml of 0.01 M phosphate buffer at pH 7.3.

**Experiment 1 Mechanism of Heparin Inhibition of Trypsin**—1 ml of trypsin solution added to 1 ml of heparin solution results in the formation of a precipitate. That small amounts of heparin markedly inhibit trypsin if the two are permitted to remain in contact with each other for definite periods of time before the addition of the protein substrate has already been demonstrated by Horwitt (2). If the mixture of the heparin and the trypsin is acidified to pH 3 or less, there is no apparent loss of activity of the trypsin after the mixture has stood for 30 minutes at room temperature and

<sup>1</sup> Heparin was obtained from The Connaught Laboratories, Toronto, hexylresorcinol from Sharpe and Dohme, Philadelphia, rennin from The Pfanstiehl Chemical Company, Waukegan, Illinois.

after subsequent testing upon a casein substrate. If, however, the trypsin-heparin mixture is permitted to stand for 10 hours at a temperature of  $25^{\circ}$ , the precipitate gradually disappears. Such a solution has no tryptic activity. If 1 ml of a fresh solution of trypsin is added to such a mixture, it combines with the heparin to form a precipitate, indicating that the heparin remained unchanged by the reactions involved during the loss of tryptic activity. The amount of the precipitate formed by mixing a trypsin solution with a given amount of heparin is proportional to the activity of the trypsin solution used. This can be proved (a) by mixing different dilutions of active trypsin with a constant amount of heparin to show that the precipitate formed in 1 minute diminishes with the enzyme concentration, and (b) by mixing heparin with a trypsin solution which has been allowed to stand at a temperature of  $30^{\circ}$  to permit the gradual formation of inactive trypsin (Northrop (1)). Inactive trypsin does not give a precipitate with heparin and the amount of heparin-trypsin precipitate formed in the latter experiment becomes progressively less as heparin is mixed with portions of trypsin solution which has been standing for longer periods of time.

It is apparent from repeated experiments that a mixture of trypsin plus heparin at pH 7.3 loses its tryptic activity on standing at a rate much more rapidly than trypsin would lose its activity in the absence of heparin. This may be due to the fact that the trypsin-heparin complex formed is changed more rapidly than trypsin itself (1).

It should be pointed out that the heparin inhibition of trypsin described by the author (2) may be quite different from that described by Glazko and Ferguson (3) who used relatively enormous amounts of heparin to obtain their inhibitions. Fischer (4) has shown that heparin can combine with casein to shift the isoelectric point of casein to the acid side, and Glazko and Ferguson used sufficient heparin to combine with an appreciable portion of the casein substrate before the protease was added.

Chymotrypsin is not inhibited by heparin (2). Solutions of this enzyme at pH 7.3 remain clear when heparin is added.

*Experiment 2 Comparison of Effects of Heparin upon Early Stages of Casein Digestion by Chymotrypsin and Trypsin*—It has been shown by the author (5) that chymotrypsin as well as relatively large amounts of trypsin can bring about the formation of a heavy turbidity in a solution of 6 per cent casein after several minutes incubation at a temperature of  $50^{\circ}$ . A study of the effect of heparin upon this reaction was made and the results are presented in Table I. 10 cc of a 6 per cent casein solution were used as a substrate. This was prepared by mixing 12 gm of casein (Hammersten) with 40 ml of 0.2 N sodium hydroxide. 20 ml of M/15 phosphate buffer at pH 7.5 plus sufficient water to bring the volume to 200 ml were

then added. The amounts of (a) chymotrypsin, (b) chymotrypsin and heparin, (c) trypsin, (d) trypsin and heparin, (e) chymotrypsin and trypsin, and (f) chymotrypsin, trypsin, and heparin, indicated in Table I, were mixed in 1 ml of  $H_2O$  and placed in the refrigerator ( $4^\circ$ ) for 30 minutes. At the end of this period the enzyme and heparin mixtures were warmed in a  $50^\circ$  water bath for 5 minutes, after which they were tipped into the 10 ml of casein solution which had also been warmed to  $50^\circ$ . Ordinary test-tubes ( $150 \times 18$  mm) were used as containers for the casein digestion. When the digestion tube reached a turbidity equal to that obtained by mixing 3 ml of a casein solution containing 3.6 mg with 3 ml of 1 per cent gum ghatti solution and 4 ml of 5 per cent sulfosalicylic acid, the time was noted.

TABLE I

*Effect of Heparin on Formation of Paracasein A by Trypsin and Chymotrypsin at pH 7.5*

The time required to reach the standard turbidity when chymotrypsin, trypsin, and mixtures of these two enzymes are added to a 6 per cent casein solution at pH 7.5 at a temperature of  $50^\circ$  was determined in the presence and absence of heparin.

Experiment a, chymotrypsin, Experiment b, chymotrypsin and heparin, Experiment c, trypsin, Experiment d, trypsin and heparin, Experiment e, chymotrypsin and trypsin, Experiment f, chymotrypsin, trypsin, and heparin.

Experiment	Enzyme concentration		Time to reach standard turbidity	
	Chymotrypsin	Trypsin	Without heparin	With 0.60 mg heparin
	mg N	mg N	min	min
a and b	0.0288		8.5	8.5
" " "	0.0480		5.2	5.0
c and d		0.0312	35.5	42.7
" " "		0.0520	22.7	27.7
e and f	0.0288	0.0312	5.0	5.8
" " "	0.0480	0.0520	2.7	3.2

(5) Table I shows that heparin had a slight inhibitory effect on the tryptic reaction rate but none at all on the chymotryptic reaction. The compound formed from casein by the protease at pH 7.3 will hereafter be referred to as paracasein A.

*Experiment 3 Comparison of Effect of Pancreatic Trypsin Inhibitor upon Formation of Paracasein A by Chymotrypsin and Trypsin*—Pancreatic trypsin inhibitor forms a compound with trypsin (Northrop and Kunitz (1)) which has no proteolytic activity. The following experiment was designed to obtain information on the effect of this inhibitor upon chymotrypsin and trypsin by using the change of casein to paracasein A as the criterion of enzymatic action. The procedure used is similar to that described above.



(Experiment 2) except that 0.60 mg of pancreatic trypsin inhibitor was substituted for 0.60 mg of heparin. The results are given in Table II. It is apparent from the data in Table II that trypsin is completely inhibited and chymotrypsin considerably inhibited by trypsin inhibitor during the initial stages of casein digestion.

*Experiment 4 Effect of Heparin and Trypsin Inhibitor on Clotting of Milk*—Since the formation of paracasein A may be similar to the production of paracasein from casein by rennet as reported by Bosworth (6) and Van Slyke and Bosworth (7), the effect of these inhibitors on milk clotting was studied. 5 ml samples of cow's milk (unbuffered, pH 6.7) were mixed with (a) 2 ml of a solution containing 1 mg of rennin, (b) 2 ml of a solution

TABLE II

*Effect of Pancreatic Trypsin Inhibitor on Formation of Paracasein A by Trypsin and Chymotrypsin at pH 8.0*

The time required to reach the standard turbidity when chymotrypsin, trypsin, and mixtures of these two enzymes are added to a 6 per cent casein solution at pH 8.0 at a temperature of 50° was determined in the presence and absence of pancreatic trypsin inhibitor.

Experiments a to f, as in Table I, with trypsin inhibitor in place of heparin

Experiment	Enzyme concentration		Time to reach standard turbidity	
	Chymotrypsin	Trypsin	Without inhibitor	With 0.60 mg inhibitor
	mg A	mg V	min	min
a and b	0.0288		12	180
" " "	0.0480		7.5	111
c and d		0.0312	68	∞
" " "		0.0520	40.7	∞
c and f	0.0288	0.0312	6.7	132
" " "	0.0480	0.0520	4.5	62.8

containing 1.0 mg of rennin and 2.5 mg of trypsin inhibitor, and (c) 2 ml of a solution containing 1.0 mg of rennin and 2.5 mg of heparin, respectively, and the time of clotting noted. The addition of these solutions to milk did not have any apparent effect on the pH of the reaction mixture. Rennin alone (a) caused a clot in 7.3 minutes, rennin plus trypsin inhibitor (b) caused a clot in 6.3 minutes, and the solution containing the rennin and the heparin (c) did not clot until 13 minutes had elapsed. Therefore, heparin is an inhibitor of rennin activity. Whether or not this inhibition is related to the known affinity of heparin for casein (4) remains to be investigated.

*Experiment 5 Effect of Hexylresorcinol upon Proteolytic Activity of Trypsin and Chymotrypsin*—That hexylresorcinol serves as a precipitant of trypsin and chymotrypsin can easily be proved by mixing solutions of these

proteases with hexylresorcinol at pH 7.3. That hexylresorcinol can cause a loss of chymotryptic activity was demonstrated in the preceding paper (5). The following experiment shows the relative effect of hexylresorcinol on the hydrolysis of casein by trypsin and chymotrypsin. The enzyme mixtures contained (a) 0.052 mg of trypsin nitrogen in 2 ml of water, (b) 0.042 mg of chymotrypsin nitrogen in 2 ml, (c) 0.052 mg of trypsin nitrogen plus 2 mg of hexylresorcinol in 2 ml, and (d) 0.042 mg of chymotrypsin nitrogen plus 2 mg of hexylresorcinol in 2 ml. These enzyme solutions stood for 1 hour in the refrigerator (10°) before they were added to 25 ml portions of the substrate which was prepared by dissolving 12 gm of casein in 8.2 ml of 1 N sodium hydroxide and diluting to a volume of 200 ml. The total volume of the mixture being digested was therefore 27 ml. At definite intervals, samples of this mixture were removed, mixed with 0.5

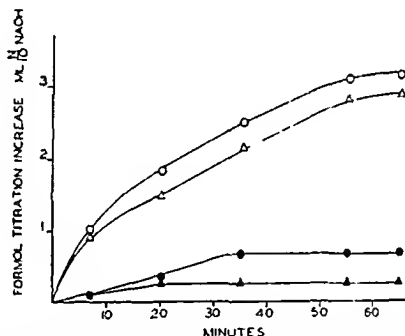


FIG 1 Effect of hexylresorcinol on digestion of casein by trypsin and chymotrypsin. ○ trypsin, △ chymotrypsin, ● trypsin plus hexylresorcinol, ▲ chymotrypsin plus hexylresorcinol

volume of 40 per cent formaldehyde, and titrated to pH 8.4 with sodium hydroxide. The increase in formol titration expressed in ml of 0.1 N sodium hydroxide per 27 ml of digestion mixtures is plotted for the different digestions in Fig 1. The curves in Fig 1 show a marked inhibition of the activity of both trypsin and chymotrypsin by hexylresorcinol.

#### SUMMARY

1. A study of precipitation reactions of trypsin and chymotrypsin with heparin, hexylresorcinol, and pancreatic trypsin inhibitor at pH 7.3 shows that trypsin is precipitated by both hexylresorcinol and heparin, that chymotrypsin is precipitated by hexylresorcinol but not by heparin, and that trypsin inhibitor precipitates neither enzyme.

2. The mechanism of trypsin inhibition by heparin is discussed.

*Extraction of Lipids*—Lipids were extracted by two methods. When a study of the lipid components of tissues or of fractions therefrom was primarily desired, the techniques of Klenk (3) were utilized. These take advantage of characteristic solubility properties of phospholipids and are applicable to material in the dry state. The proteins undergo denaturation during such extraction. When it was of importance to study the behavior of extracted but undenatured proteins, the Hardy-Gardiner method (4, 5) of lipid extraction, which is carried out in the cold and produces no observable alteration of the protein, was employed.

*Preliminary Observations*—Although powders were prepared from spleen by a standardized procedure, the coagulative potency of saline extracts of different lots was not uniform. Usually clotting was accelerated, but some extracts did not influence the coagulation time, and only occasional preparations delayed or prevented coagulation of test plasmas. The anticoagulant activity could not be correlated either with the concentration of saline (0.9 to 2.5 per cent) used for the extraction of the dry powder or with the total protein concentration of the extract. When increased quantities of anticoagulant extracts of spleen were added to plasma, progressive retardation and in some instances complete prevention of coagulation occurred. Dialysis did not influence the anticoagulant activity. When the extracts were boiled, or were treated with trichloroacetic or tungstic acid, precipitation occurred and the supernatant became inactive. Acidification (pH 3.5) also precipitated proteins and left the supernatant inactive but the redissolved, neutralized, and dialyzed protein retained the anticoagulant property, provided the treatment with acid was of only short duration.

*Fractionation of Proteins*—The above observations suggested that anticoagulant activity was associated with a protein component of the extract. Purification of the anticoagulant by protein fractionation was prompted by the desire to eliminate interfering tissue thromboplastin, which separates with the most readily precipitable globulin fractions (6). The proteins of tissue extracts were fractionated with ammonium sulfate. The globulins were collected by centrifugation after 33 and 50 per cent saturation and the albumins after complete saturation with ammonium sulfate. The fractions were dialyzed in Visking casings against frequent changes of physiological saline until the outside fluids were free from sulfate. The albumin fractions formed clear, transparent, deep red solutions. The relative activity of the three protein fractions of an extract obtained from pig spleens is illustrated in Table I.

As is seen in Table I, the two globulin fractions accelerated and the albumin fraction inhibited the coagulation, in the whole extract the activity of the globulins was almost entirely masked by that of the albumin. Identical results were obtained with other tissue extracts, regardless of their origin and initial activity.

In addition to extracts of spleen, extracts of lung, testicle, kidney, liver, muscle, and red blood cells were examined. Salts, other than ammonium sulfate, which permit the separation of albumins from globulins by salting-out, i.e.  $\text{MgSO}_4$ ,  $\text{Na}_2\text{SO}_4$ , can be employed to separate anticoagulant albumins. The anticoagulant activity, however, is not quantitatively related to the protein concentration of a given tissue albumin solution. Indeed some albumin fractions, as for example those separated from dog plasma, are entirely negative in this respect. Evidently the anticoagulant quality is indicative of a specific type of albumin. Elimination of inactive albumins was achieved by isoelectric precipitation of the anticoagulant in the presence of ammonium sulfate.

*Fractionation of Anticoagulant Tissue Albumins*—Anticoagulant tissue albumin was obtained from an extract of spleen by three repeated precipitations with ammonium sulfate. To a 3 per cent aqueous solution of this albumin solid ammonium sulfate was added to 50 per cent saturation. This

TABLE I  
*Fractionation of Saline Extract of Spleen with  $(\text{NH}_4)_2\text{SO}_4$*

Concentration of $(\text{NH}_4)_2\text{SO}_4$	Protein concentration of dialyzed fractions	Coagulation time of test plasma with 0.2 ml of each fraction
<i>per cent</i>	<i>per cent</i>	<i>sec</i>
33	0.86	134
50	1.14	245
100	1.65	>1800
Original extract of spleen	3.86	342
Saline controls		375

caused no precipitation. The pH was then adjusted to 3.7 by the addition of  $\text{N H}_2\text{SO}_4$  (Diagram 1) and the gray precipitate was promptly collected by centrifugation. The clear supernatant was saturated with ammonium sulfate, the precipitate was designated Fraction A. The gray acid precipitate was treated at pH 7.2 with a volume of distilled water equal to approximately one-half that of the original albumin solution. The residue was designated Fraction B. The redissolved portion was acidified to pH 3.7 and the resulting precipitate removed by centrifugation without delay. The clear supernatant on full saturation with ammonium sulfate furnished Fraction C. The precipitate that separated at pH 3.7 was again treated with water at pH 7.2 and the solute reprecipitated at pH 3.7, yielding Fraction D. All fractions were dialyzed against physiological saline until they were free of ammonium sulfate. Each fraction was then brought to a uniform protein concentration of 0.3 per cent and tested for anticoagulant activity. Fraction A only retarded but did not prevent the coagulation of test plasmas. No coagulation occurred for more than 1 hour when 0.2 ml

Table II, the alcohol-chloroform extract was anticoagulant. The residual protein was soluble in saline but had lost its anticoagulant activity and yielded none to ether. The results with the Hardy-Gardiner method of extraction are in accord with the known solubility characteristics of sphingomyelins. The inertness of the residual protein suggests that it is only the carrier of an active anticoagulant prosthetic group.

*Extraction and Purification of Sphingomyelins of Tissues*—Anticoagulant sphingomyelins were isolated from tissue powders by a slightly modified Klenk procedure (3). It was of interest that sphingomyelins extracted by acetone on dehydration of tissues were not anticoagulants. Dry tissue

TABLE II

*Extraction of Anticoagulant Tissue Albumin by Modified Hardy-Gardiner Procedure at  $-14^{\circ}$*

Clotting times of recalcified plasmas upon the addition of 0.2 ml. of each fraction are recorded. The tissue albumin solution is the same as that used in preceding experiments of Hardy-Gardiner extraction.

Saline controls		Tissue albumin solution		
Before extraction				
sec				
405		In 1800 sec viscous, semisolid coagulation		
After extraction				
Experiment No	Saline controls	Ethanol chloroform extract	Soxhlet ether extract	Extracted albumin
	sec		sec	sec
I	405	No change in 960 sec , later in one tube fibrin-fibers, others free from coagulation for more than 1800 sec	402	525
II	295	Gelatinous in about 720 sec , no fibrin separation for more than 1800 sec	300	450

powders, in 150 gm. lots, were extracted five successive times with ether in the Soxhlet apparatus. Thereafter the residue was further extracted eight times with boiling absolute ethanol and five times with boiling chloroform-methanol (3:1) mixture. The alcoholic extracts were filtered while hot. When cooled in the ice chest, crude sphingomyelins and cerebrosides separated as amorphous, red precipitates. A total of 28.8 gm. of this fraction was collected from spleen powders in an over-all yield of 0.86 per cent. Additional crops of this fraction weighing 2.08 gm. were obtained by (1) cooling the mother liquors to  $-14^{\circ}$  and (2) concentration of the supernatants *in vacuo* and precipitation with acetone. All the crude sphingomye

lins obtained by the above methods contained sulfur and were, as is shown in Table III, anticoagulants. The purification of anticoagulant sphingomyelins was accomplished by thoroughly washing with ether and repeatedly precipitating from chloroform-methanol (3:1), ethyl acetate, glacial acetic acid, and warm pyridine with the aid of acetone. After twenty-six precipitations 0.56 gm of a red-brown, scaly material was obtained that showed all the solubility characteristics of sphingomyelins. Analysis<sup>2</sup> showed N 3.27 per cent, P 2.35 per cent, sulfur was absent. The product was observed to swell in water or saline to form myelin figures and to yield faintly yellow-colored, clear solutions. It was a more potent anticoagulant than the crude preparation (Table III). The analysis indicated that cerebroside were still present in the final preparation, since the acid hy-

TABLE III

*Effect of Anticoagulant Sphingomyelin on Plasma Coagulation, Clotting Times of Recalcified Plasmas*

Saline controls clotting times	Crude sphingomyelin in saline	Clotting times
0.2 ml, 290 sec	25 mg in 0.1 ml	660-720 sec, flabby, viscous
	50 " " 0.2 "	1260 sec viscous, 1740 sec fine fibrin veil
	75 " " 0.4 "	1560 sec viscous, 1860 sec fibrin veil, 2040 sec flabby viscous <i>in toto</i>
Purified sphingomyelin in saline		
0.1 ml, 298 sec	0.2 mg in 0.1 ml	500
0.2 " 301 "	0.4 " " 0.2 "	900
0.4 " 290 "	0.8 " " 0.4 "	>1800

drolysate of the product gave a positive Molisch reaction. The cerebroside, however, separated during the purification process, were completely devoid of anticoagulant activity.

#### DISCUSSION

Saline extracts of tissues contain at least two distinct fractions which may antagonistically influence the coagulation of recalcified plasma. That which accelerates coagulation and is associated with tissue globulins is known as thromboplastin. Its active lipid component was recognized by Howell and McLean as cephalin (7, 8). Evidence is presented in this report that another lipid-protein complex which delays or prevents coagulation is associated with the tissue albumins. Its active lipid component

<sup>2</sup> Analyses were made by Mr. J. F. Alicino.



THE CHEMISTRY OF THE LIPIDS OF TUBERCLE BACILLI  
LXX CONCERNING THE DEXTROROTATORY FATTY ACIDS OF THE  
ACETONE-SOLUBLE FAT OF CELL RESIDUES FROM THE  
PREPARATION OF TUBERCULIN\*

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In a study of the fatty acid components of the tubercle bacillus phosphatide one of us isolated a new liquid saturated, optically active fatty acid of high molecular weight (1). The physiological activity of the acid was investigated by Sabin and collaborators (2) and it was found that on injection into normal animals the acid stimulated proliferation of monocytes, epitheloid and giant cells with the formation of typical tubercular tissue. In view of its origin and on account of its peculiar physical properties and unusual biological activity the acid was named phthioic acid (3).

The molecular weight of different preparations of the acid varied from 306 to 313 and the specific optical rotation was about  $+1.5^\circ$ . In the light of the later work it is evident that the original acid must have been a mixture of tuberculostearic acid and phthioic acid.

Larger quantities of analogous liquid saturated fatty acids were later isolated by Anderson and Chargaff (4) from the acetone soluble fat of the human tubercle bacillus, Strain H-37. By fractional distillation of the methyl ester two different acids were obtained. The lower boiling fraction of the ester gave on saponification an optically inactive liquid saturated acid that corresponded approximately in composition to stearic acid, and hence was named tuberculostearic acid. It was shown later by Spielman (5) that tuberculostearic acid had the formula  $C_{19}H_{38}O_2$  and that its structure was 10-methylstearic acid. Tuberculostearic acid did not show any biological activity.

The higher boiling ester gave on saponification an acid which had a specific rotation of about  $+8.0^\circ$  and which corresponded in composition to a saturated hexacosanoic acid,  $C_{26}H_{52}O_2$ . The name phthioic acid was applied to this acid because, as shown by Sabin and collaborators (6), it gave biological reactions similar to the acid prepared from the phosphatide.

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† Holder of a National Tuberculosis Association Fellowship at Yale University, 1942-44.



In a later investigation by one of us (7) a mixture of the methyl esters of liquid saturated fatty acids obtained from the tubercle bacillus, Strain H-37, was fractionated into three principal components (a) the optically inactive ester of tuberculostearic acid, (b) the dextrorotatory ester of phthioic acid, and (c) the ester of a levorotatory acid to which the provisional formula  $C_{30}H_{60}O_2$  was assigned. The purest phthioic acid obtained in this experiment had a specific dextrorotation of  $+11.96^\circ$  and its composition corresponded to the formula  $C_{26}H_{52}O_2$ .

Spielman and Anderson (8) reported a study on phthioic acid and these authors fractionated methyl phthioate until it showed the constant specific rotation of  $+12.2^\circ$ . In view of the fact that various fractions of the purified ester showed the same constant rotation, the ester was regarded as homogeneous. The free phthioic acid obtained on saponification of the ester was a thick oil at room temperature which solidified on cooling, and melted at  $20-21^\circ$ , and the specific rotation was  $+12.56^\circ$ . The analysis of the ester, the free acid, and its silver salt agreed with the formula  $C_{26}H_{52}O_2$  for the acid. The physiological activity of this highly purified phthioic acid was tested in Dr. Sabin's laboratory and it was found to give the same cellular reactions as the less pure acids that had been tested previously.

In view of the physiological activity and unusual properties of phthioic acid, its chemical structure has presented a problem of great interest, but up to the present time its constitution has not been established. The oxidation and degradation experiments reported by Spielman and Anderson (8) did not lead to any definite conclusions concerning the structure of phthioic acid, but their results indicated that the acid possessed a branched chain structure and that a methyl group was probably attached to the  $\alpha$ -carbon and another in the neighborhood of the 11th carbon atom, but possibly other branches were also present.

A number of branched chain fatty acids have been synthesized in recent years in attempts to prepare model molecules for comparison with phthioic acid, but the properties of the synthetic acids so far described differ from those of the natural phthioic acid. Chargaff (9) synthesized a series of  $\alpha$ -substituted hexacosanoic acids. Birch and Robinson (10) prepared a number of long chain substituted acetic and propionic acids. Schneider and Spielman (11) synthesized a series of  $\alpha$ -methyl and 10 methyl substituted fatty acids, containing from 19 to 27 carbon atoms.

Cason (12) and Cason and Prout (13) have reported the synthesis of some new branched chain acids. Other attempts to synthesize fatty acids with properties similar to those of phthioic acid have been reported by Buu-Hoi and Cagniant (14). These investigators claim that  $\alpha, \alpha$ -dimethyl myristic acid,  $\alpha, \alpha$ -dimethylpalmitic acid, and  $\alpha, \alpha$ -dimethylstearic acid

on intraperitoneal injection caused an increase in monocyte count and give characteristic lesions

On the basis of the properties of surface films of phthioic acid in comparison with similar determinations with branched chain acids of known constitution Stenhagen and Stallberg (15) suggested that phthioic acid might be a trisubstituted acetic acid. A series of trisubstituted acetic acids corresponding to the model suggested by Stenhagen and Stallberg was synthesized by Polgar and Robinson (16) but the properties of these synthetic acids were found to differ from those of phthioic acid. The same authors (16) report the synthesis of other branched chain acids which, however, all differed in properties from phthioic acid.

Wagner-Jauregg (17) investigated some liquid saturated fatty acids prepared from the acetone-soluble fat from cell residues from the manufacture of tuberculin. The methyl esters of the liquid saturated acids were separated by distillation into low boiling and high boiling fractions. The low boiling fractions yielded on saponification an acid corresponding to tuberculostearic acid, while the high boiling fractions gave on saponification an acid called "phthioic acid". From the results reported it is evident that the acids examined by Wagner-Jauregg could not have contained any phthioic acid because both the ester and the free acid were found to be *optically inactive*. The esters of both acid fractions were oxidized by the Kuhn-Roth method (18). The methyl tuberculostearate gave 1.4 moles of acetic acid. The higher boiling ester gave 2.32 and 2.41 moles of acetic acid, thus indicating the presence of possibly three terminal methyl groups. Since the material that was examined was optically inactive, the data reported can have no direct bearing on the number of methyl groups or branches in the chain of the dextrorotatory phthioic acid which occurs in the human tubercle bacillus, Strain H-37.

The strain of bacilli examined by Wagner-Jauregg was not given, but evidently human strains of tubercle bacilli had not been used. It has been found in this laboratory that the acetone-soluble fats extracted from several strains of the human tubercle bacillus that have been cultivated on the Long synthetic medium all contain optically inactive tuberculostearic acid and dextrorotatory acids corresponding in properties to phthioic acid.

In recent studies in this laboratory it has been found, however, that the human tubercle bacillus, Strain H-37, when cultivated on a modified Long medium in which dextrose was the chief source of carbon, elaborates different lipids than when glycerol constitutes the main source of carbon (19). Furthermore, as recorded in Paper LXIX of this series (20), an unidentified strain of the tubercle bacillus which had been grown for the production of a special lot of the purified tuberculin protein, PPD, yielded

an acetone-soluble fat from which several different liquid saturated fatty acids were isolated

The present report deals with the composition of the high boiling dextrorotatory ester fractions obtained from the above mentioned acetone soluble fat. The properties of the esters resembled those of crude methyl phthioate, but on careful fractionation four apparently pure but different fractions were obtained that varied in composition and in the magnitude of optical rotation. The esters were saponified and the free acids were isolated. The acids were thick oils at ordinary room temperature and their lead salts were easily soluble in ether. In composition the acids corresponded to the formulas  $C_{21}H_{42}O_2$ ,  $C_{25}H_{50}O_2$ ,  $C_{25}H_{52}O_2$ , and  $C_{27}H_{54}O_2$ . The rotations varied from  $+5.17^\circ$  to  $+17.11^\circ$ .

The four acid fractions were analyzed for methyl groups attached to carbon by a modified Kuhn-Roth method. In standardizing this procedure synthetic branched chain fatty acids of known constitution were first analyzed and it was found that terminal methyl groups were oxidized to acetic acid in yields of from 80 to 90 per cent. The yield of acetic acid on oxidation of the dextrorotatory acids ranged from 2.4 to 2.7 moles per mole of acid. It may be concluded, therefore, that at least three terminal methyl groups were present in each acid.

The acid that corresponded to the formula  $C_{26}H_{52}O_2$  was identical in melting point and optical rotation with the purified phthioic acid described by Spielman and Anderson (8). The other acids, however, showed such marked differences in composition and in optical rotation that we must conclude that they represent a series of acids that differ by 1 and 2 carbon atoms from phthioic acid, and that they must possess different constitutions due to variations in length of the branches, and to their position along the chain.

The results of our recent observations serve to emphasize the importance of the composition of the culture medium as well as of the strain of bacilli upon the nature and chemical structure of certain metabolic products produced by the human tubercle bacillus under different conditions of cultivation. In future investigations these facts must be considered, since variation in chemical composition of specific metabolic products may be as common as variation in virulence of different strains of tubercle bacilli.

#### EXPERIMENTAL

The high boiling dextrorotatory ester fractions used in this investigation had been obtained from the acetone-soluble fat of cell residues from the preparation of the purified protein, PPD, as described in Paper LXIX of this series (20). The observed specific rotations of the ester fractions

varied from  $+10.3^\circ$  to  $+11.9^\circ$ . These values are only slightly lower than the constant rotation of  $+12.2^\circ$  reported by Spielman and Anderson (8) for the pure methyl ester of phthalic acid. It appeared probable therefore that the esters consisted mainly of methyl phthalate.

*Fractionation of the Esters*—For further purification the ester fractions were combined, total weight about 25 gm, and subjected to a series of fractionations through a special column designed by Dr S F Velick of this laboratory. In a preliminary experiment 4.06 gm of the ester were distilled at a pressure of 1 to 2 mm and six fractions and a residue were collected. The results are shown in Table I.

It will be noted that the ester fractions showed a steady increase in refractive index which is characteristic of a mixture. The optical rotation rose to a maximum, descended to a minimum, again rose to a maximum,

TABLE I

*Distillation of 4.06 Gm of Crude Dextrorotatory Esters at a Pressure of 1 to 2 Mm*

Fraction No	Pot t	Column t	Weight	$n_D^{25}$	$[\alpha]_D^{25}$
	C	C	mg		degrees
1	208	194	280	1.4548	+7.33
2	210	198	512	1.4569	+11.95
3	210	199	455	1.4586	+12.51
4	213	203	621	1.4588	+11.04
5	217	206	788	1.4596	+11.59
6	220	210	801	1.4610	+12.99
Residue			584	1.4627	+6.45

The total ester fractions recovered amounted to 4.04 gm

and then decreased rapidly. The balance of the crude esters was distilled under the same conditions in three equal portions with almost identical results as those shown in Table I. A composition curve was plotted from the values of refractive index and optical rotation. The shape of the optical rotation curve indicated that the starting material was a mixture of at least four components.

*Purification of the Ester Fractions*—The fore runs from the preliminary distillations were combined and refractionated several times until an apparently homogeneous fraction was obtained which on further distillation showed no appreciable variation in refractive index or in specific optical rotation. In like manner the fractions from the first maximum, from the intermediate minimum, and from the second maximum of the optical rotation curve were combined and refractionated repeatedly until fractions showing constant properties were obtained. A separation into

four components was thus achieved. The properties of the purified esters are given in Table II.

Although the physical properties of the four ester fractions remained constant on repeated fractionations, this is no absolute proof of purity because criteria of purity for compounds of this type do not exist at present. The results indicate nevertheless that apparently four different components were present in the original ester mixture. Only about 30 per cent of the starting material was accounted for by the four purified components, the balance represents intermediate fractions remaining from the numerous redistillations.

*Isolation of the Free Acids*—The four ester fractions were saponified separately by refluxing for several hours with an excess of alcoholic potassium hydroxide, after which the free acids were isolated in the usual manner. For purification the free acids were dissolved in alcohol and the solutions were neutralized with alcoholic potassium hydroxide, after which

TABLE II  
Properties of the Purified Esters

Component No	Distillation at 1 to 2 mm pressure		Weight	$n_D^{25}$	$[\alpha]_D^{25}$	M p
	Pot t	Column t				
	C	C	gm		degrees	C
1	197	184	1.4	1.4508	+4.4	17-18
2	207	196	1.3	1.4576	+12.6	11-12
3	212	201	2.4	1.4579	+10.1	15-16
4	216	204	2.6	1.4605	+13.2	13-14

an alcoholic solution of calcium chloride was added in excess. The calcium salts which separated as white amorphous powders were filtered off and washed thoroughly with alcohol in order to remove any unsaponified ester.

The calcium salts were suspended in ether and decomposed by shaking with dilute hydrochloric acid. The ethereal solutions were washed with water until the washings were neutral, dried over sodium sulfate, filtered, and the ether was removed by distillation. The acids were dried *in vacuo* over sulfuric acid.

For analysis the acids were dried to constant weight at 78° over diphosphoric acid. The silver salts were prepared and analyzed as described by Anderson and Chargaff (4). The molecular weights were determined by titration in benzene solutions with standard alcoholic potassium hydroxide. The acids were also analyzed for methyl groups attached to carbon by a slightly modified Kuhn-Roth procedure as will be described in a following paper.

The physical properties of the acids and the analytical results are recorded in Table III

The data presented in Table III show that the analytical values agree with the formulas  $C_{24}H_{48}O_8$ ,  $C_{25}H_{50}O_8$ ,  $C_{26}H_{52}O_8$ , and  $C_{27}H_{54}O_8$ . However, as mentioned previously no absolute homogeneity can be claimed. Since the analyses for carbon and hydrogen cannot differentiate between higher fatty acids that differ by only 1 carbon atom, the silver salts were also prepared and analyzed. It will be noted that the composition of the silver salts agrees closely with the values for molecular weights determined by titration.

Attention is called to the fact that the acid listed as Component 3 in Table III corresponds in properties to phthioic acid. Spielman and

TABLE III  
*Properties of Acids*

Com ponent No	M p	$n_D^{25}$	$[\alpha]_D^{25}$	Equi valent weight by titra tion	Proposed formula	Elementary analysis				Silver salt		Acetic acid by Kuhn Roth oxida tion Found		
						C		H		Calcu lated	Found		Calcu lated	Found
						Calcu lated	Found	Calcu lated	Found					
	C		degrees			per cent	per cent	per cent	per cent	per cent Ag	per cent Ag	moles		
1	23-24	1.4565	+5.17	371	$C_{24}H_{48}O$ (368)	78.25	78.19	13.04	12.97	22.71	22.66	2.43		
2	17-18	1.4642	+15.60	383	$C_{25}H_{50}O_2$ (382)	78.53	78.91	13.08	12.97	22.06	22.04	2.63		
3	21-22	1.4643	+12.78	397	$C_{26}H_6O$ (396)	78.79	79.05	13.13	12.92	21.45	21.36	2.68		
4	20-21	1.4671	+17.11	407	$C_{27}H_7O_2$ (410)	79.02	79.30	13.17	13.08	20.86	20.94	2.71		

Anderson (8) assigned the formula  $C_{26}H_{52}O_8$  to phthioic acid and reported the following constants: m.p. 20-21°,  $n_D^{25}$  1.4628, and  $[\alpha]_D^{25}$  +12.56°. These values are almost identical with those found for Component 3.

The values found in the Kuhn-Roth oxidation indicate that each of the acid fractions contains at least three terminal methyl groups. Thus the acids likely are molecules with doubly branched carbon chains, but the positions and length of the chains are unknown. The variations in optical rotation, however, indicate that the branches are located at different positions.

#### DISCUSSION

The results reported in this paper might cast some doubts upon the purity and homogeneity of phthioic acid because a mixture of the four

component acids listed in Table III would approximately correspond to the formula  $C_{26}H_{50}O_2$ . This formula was originally assigned to phthioic acid (4) and confirmed by the work of Spielman and Anderson (8). The latter authors made every effort to purify the methyl ester of phthioic acid as carefully as possible by the methods available at that time.

The question of the homogeneity of phthioic acid isolated from the human tubercle bacillus, Strain H-37, cannot be settled at the present time, due to lack of material. In order to settle this matter it would be necessary to secure new lots of tubercle bacillus, Strain H-37, for the purpose of preparing a sufficiently large quantity of the dextrorotatory acid to permit of its thorough purification.

As mentioned previously the dextrorotatory fatty acids used in the present investigation were obtained from an unidentified strain of the tubercle bacillus. It is possible that this strain produced different acids from Strain H-37. We hope that the investigation may be continued when additional quantities of Strain H-37 become available.

The authors wish to express their thanks to Dr. L. H. Chang for carrying out some of the analyses.

#### SUMMARY

1. An examination has been made of the dextrorotatory fatty acid fraction isolated from the acetone soluble fat of an unidentified strain of tubercle bacilli residues which remained from the preparation of the purified protein PPD.

2. By repeated fractionations of the methyl esters four different acids were isolated which differed in specific optical rotation and in molecular weight.

3. The purified acids corresponded to the formulas  $C_{24}H_{48}O_2$ ,  $C_{25}H_{50}O_2$ ,  $C_{26}H_{52}O_2$ , and  $C_{27}H_{54}O_2$ .

4. The properties of the acid having the formula  $C_{26}H_{50}O_2$  agree with those reported for phthioic acid.

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# THE CHEMISTRY OF THE LIPIDS OF TUBERCLE BACILLI

## LXXXI THE DETERMINATION OF TERMINAL METHYL GROUPS IN BRANCHED CHAIN FATTY ACIDS\*

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In the course of an investigation on optically active branched chain fatty acids derived from the acetone-soluble fat of tuberculin residues as described in the preceding paper (1), it became of interest to determine terminal methyl groups, since such data would give an indication of the number of branches in the molecules of the acids. An attempt was first made to determine the acetic acid formed on the oxidation of stearic acid and *n*-hexacosanoic acid by the Kuhn-Roth (2) method according to the directions of Roth and Daw (3). The results were low and irregular, apparently due to the incomplete oxidation of the acids caused by their low solubility in the oxidizing mixture. A slight modification in the procedure, however, led to more satisfactory results with yields of acetic acid from about 80 to 90 per cent of the calculated value.

The new procedure was applied in the analysis of branched chain fatty acids of known structure and from 75 to 85 per cent of the theoretical value for acetic acid was found. Under the same conditions the dextro-rotatory fatty acids isolated from the acetone soluble fat of tuberculin residues (1) gave from 2.4 to 2.7 moles of acetic acid, thus indicating that these acids contained at least three terminal methyl groups.

### EXPERIMENTAL

#### *Reagents—*

Concentrated c.p. sulfuric acid

5 N chromic acid solution

0.00956 N sodium hydroxide

20 per cent solution of hydrazine hydrate

Recently boiled distilled water

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### Procedure

The apparatus described by Kuhn and Roth (2) was used according to the directions of Roth and Daw (3)

For the analyses, from 10 to 20 mg of fatty acid were dissolved in 2 cc of concentrated sulfuric acid by warming. After the solution had cooled to room temperature, a few drops of the chromic acid solution were added until the oxidation began, after which the solution was cooled in ice water and 5 cc of the chromic acid solution were added. The solution was then refluxed for 90 minutes, since it had been found in preliminary experiments that refluxing the reaction mixture for 90 minutes was adequate for the complete oxidation of the fatty acids.

After the solution had been cooled to room temperature, the inside of the condenser was carefully washed into the reaction flask with  $\text{CO}_2$  free water, and the excess chromic acid was destroyed by the addition of

TABLE I

*Determination of Terminal Methyl Groups in Normal Fatty Acids*

Time of oxidation 90 minutes

Substance	Weight of sample	0.00956 N NaOH		$\text{CH}_3\text{COOH}$ per mole fatty acid Found
		Calculated for $1\text{CH}_3$	Found	
	mg	cc	cc	mole
$\text{CH}_3\text{CO}_2\text{Na} \cdot 3\text{H}_2\text{O}$	11.76	9.03	8.82	0.98
Stearic acid	18.80	6.91	6.31	0.91
" "	19.61	7.22	6.68	0.92
Hexacosanoic acid	21.64	5.69	4.64	0.81

12 drops of 20 per cent hydrazine hydrate. The total volume of the solution measured about 25 to 30 cc. Instead of neutralizing the solution with sodium hydroxide and acidifying with phosphoric acid as recommended by Kuhn and Roth, we distilled off the acetic acid directly and titrated the distillates with 0.00956 N sodium hydroxide with phenolphthalein as indicator. The distillates were collected in 5 cc portions as described by Kuhn and Roth, and after each collection 5 cc of water were added to the reaction flask through the stoppered funnel. It was necessary to distil off fifteen 5 cc portions in order to remove all of the acetic acid. The distillates were tested with barium chloride in the usual manner and were found to be free from sulfuric acid. Slight bumping during the distillation could be controlled by means of an ebulator. The results obtained are presented in Tables I, II, and III.

The data presented in Table I show that oxidation of normal fatty acids such as stearic acid and hexacosanoic acid yield from 80 to 90 per cent

TABLE II

*Determination of Terminal Methyl Groups in Branched Chain Fatty Acids of Known Constitution*

Time of oxidation 90 minutes

Substance	Weight of sample	0.00956 N NaOH		CH <sub>3</sub> COOH per mole fatty acid found
		Calculated for 1CH <sub>3</sub>	Found	
	mg	cc	cc	moles
$\alpha$ -Ethylbutyric acid	10.48	9.44	13.56	1.44
$\alpha$ -Methylstearic "	14.59	5.12	8.46	1.65
$\alpha$ -Methyltricosanoic acid	14.92	4.78	7.82	1.64
$\alpha$ -Methyldocosanoic "	15.90	4.69	7.77	1.66
$\alpha$ -Methyltetracosanoic acid	16.15	4.42	7.14	1.62
$\alpha$ -Methylhexacosanoic "	15.46	3.94	6.50	1.85
10-Methylstearic acid	14.26	5.00	8.06	1.61
10-Methyldocosanoic acid	15.72	4.64	7.54	1.63
10-Methyltetracosanoic acid	16.91	4.62	6.66	1.45
10-Methylhexacosanoic "	20.46	5.21	7.51	1.44
15-Methyloctadecanoic "	10.15	3.56	6.51	1.84
16-Methyloctadecanoic "	9.11	3.19	4.53	1.42
Tuberculoic acid	13.97	4.90	8.95	1.83
" "	15.07	5.29	9.69	1.83

TABLE III

*Determination of Terminal Methyl Groups in Branched Chain Fatty Acids of Unknown Constitution*

Time of oxidation 90 minutes

Substance	Weight of sample	0.00956 N NaOH		CH <sub>3</sub> COOH per mole fatty acid found
		Calculated for 1CH <sub>3</sub>	Found	
	mg	cc	cc	moles
Phytomonic acid	14.95	5.01	8.22	1.64
Dextrorotatory fatty acids from tuberculin residues				
C <sub>24</sub> acid	15.74	4.47	10.80	2.42
" "	11.31	3.21	7.89	2.45
C <sub>25</sub> "	12.57	3.44	9.25	2.68
" "	12.59	3.45	8.86	2.58
C <sub>26</sub> "	11.88	3.13	8.57	2.73
" "	12.86	3.39	8.95	2.64
C <sub>27</sub> "	12.30	3.14	8.41	2.68
" "	12.06	3.08	8.43	2.74

of the calculated amount of acetic acid. A control determination on sodium acetate gave practically quantitative recovery of acetic acid.

In Table II data are presented on a series of  $\alpha$ -methyl substituted acids

from  $C_{19}$  to  $C_{-7}$ , all of which gave about 1.64 moles or about 82 per cent of the calculated amount of acetic acid. A series of miscellaneous synthetic fatty acids with methyl groups in various positions along the chain gave somewhat variable recoveries of acetic acid ranging from 75 to 85 per cent. It will be noted that synthetic 10-methylstearic acid and the natural tuberculostearic acid, which was shown by Spielman (4) to have the configuration of 10-methylstearic acid, both gave values similar to the other monosubstituted fatty acids. Although the results are not quantitative, the data indicate that the method is applicable to the determination of the approximate number of methyl groups in branched chain fatty acids.

The data presented in Table III show that the dextrorotatory fatty acids isolated from the acetone-soluble fat of tuberculin residues (1) gave values ranging from 2.42 moles to 2.74 moles of acetic acid. If we assume that the terminal methyl groups in these acids are converted into acetic acid in yields of from 80 to 90 per cent, the results would indicate that these acids contain three terminal methyl groups. It seems likely, therefore, that these acids have doubly branched carbon chains, a configuration suggested by Spielman and Anderson (5) for phthioic acid.

Phytomonic acid, a branched chain  $C_{20}$  acid isolated by Velick (6) from *Phytomonas tumefaciens*, yielded 1.64 moles of acetic acid. This value indicates the presence of two terminal methyl groups in the molecule of phytomonic acid which is in agreement with the singly branched structure proposed by Velick (7).

#### SUMMARY

1 The Kuhn-Roth method for the determination of methyl groups attached to carbon has been slightly modified and applied to the determination of terminal methyl groups in branched chain fatty acids.

2 In the case of branched chain fatty acids of known structure the yield of acetic acid ranged from 75 to 85 per cent of the calculated values.

3 The application of the analysis to the dextrorotatory fatty acids of unknown structure isolated from the acetone-soluble fat of tuberculin residues indicates that these acids contain doubly branched chains.

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# THE COLORIMETRIC ESTIMATION OF SMALL AMOUNTS OF AMMONIA BY THE PHENOL-HYPOCHLORITE REACTION

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The micro diffusion technique of Conway (1) is a satisfactory procedure for the isolation of small quantities of ammonia. The estimation of very small amounts of ammonia by the usual back titration offers some difficulties, since it requires the use of special ultramicro burettes, extensive precautions against contamination with carbon dioxide and other acids and bases, and considerable experience with the method on the part of the operator. A simple and sensitive colorimetric method for ammonia has been elaborated as an alternative procedure. The reaction, first used extensively by Van Slyke and Hiller (2) and by Borsook (3), is that which occurs between ammonia, phenol, and hypochlorite in alkaline solution to yield an intense blue product (believed to be indophenol or a closely related substance). Further study of the reaction has led to a several fold increase in its sensitivity. With a photoelectric colorimeter, it is possible to detect 0.1  $\gamma$  per ml. and to determine with reasonable accuracy 0.5  $\gamma$  per ml. or more of ammonia nitrogen.

## Reagents—

1 Alkaline phenol reagent. 25 per cent phenol in 2.7 *N* sodium hydroxide. Mix 25 gm. of crystalline phenol with water, add with stirring 54 ml. of 5.0 *N* sodium hydroxide and make to 100 ml. Preserve in a brown bottle in the refrigerator.

2 Hypochlorite solution. Grind and sift 25 gm. of calcium hypochlorite (bleaching powder, U. S. P.) and dissolve it as far as possible in 300 ml. of hot water. Add with stirring 135 ml. of potassium carbonate solution (20 gm. of anhydrous salt in 100 ml. of solution, previously boiled to free it of ammonia). Mix thoroughly, heat briefly to about 90°, cool, and make to 500 ml. Filter a small portion of the mixture and test for calcium ion, if the test is positive, add more carbonate to the mixture until a negative test is obtained. Filter the mixture and store the filtrate in small brown bottles in the refrigerator. This solution should be water-clear and contain 1.30 to 1.40 gm. of free chlorine per 100 ml.

*Test for Calcium Ion*—To 1 ml. of the solution add a little of the potassium carbonate solution and heat in boiling water a few minutes. The solution should remain crystal-clear in the absence of calcium ion.

*Test for Free Chlorine*—To 200 ml of the hypochlorite solution add 10 ml of water, 2 ml of 5 per cent potassium iodide, and 1 ml of glacial acetic acid. Titrate with 0.100 N sodium thiosulfate (starch indicator), 7.5 to 8.0 ml of thiosulfate should be required. Retest the strength of the solution occasionally.

3. Manganese salt. Manganous chloride or sulfate 0.003 M

*Color Development*—Place in a calibrated test-tube or colorimeter tube 1.5 ml of sample containing 0.5 to 6  $\gamma$  of ammonia N, 1 drop (about 0.05 ml) of 0.003 M manganous salt solution, 1 ml of alkaline phenol reagent, and 0.5 ml of hypochlorite solution. The two latter solutions should be cold when added to the sample, loss of ammonia from the alkaline solution may be further reduced by keeping the sample tubes in an ice bath during their preparation. Mix the contents of the tubes by gentle rotation, and place them immediately in a briskly boiling water bath for about 5 minutes. Cool. Make to a convenient volume (e.g., 6 or 10 ml) and read in a photoelectric colorimeter with a filter having an absorption maximum near 625 m $\mu$ .

The sample volume may be as much as 5 ml if larger amounts of nitrogen are present. In this case the amounts of manganese and hypochlorite added should be increased approximately in proportion to the total volume of the solution during color development. Standards and blanks must of course be prepared to have the same composition. With the volumes given above and a volume at a reading of 6 ml, 0.1  $\gamma$  of nitrogen gives a detectable color and 0.5 to 6  $\gamma$  may be determined. With a sample volume of 5 ml, 1 ml of phenol reagent and 1 ml of hypochlorite solution and a final volume at a reading of 10 ml, 2 to 20  $\gamma$  of nitrogen may be determined.

The ammonia sample should be neutral or in acid not more than 0.01 to 0.02 N in strength. When the method is used in conjunction with the Conway micro diffusion technique, the contents of the inner wells of the absorption units are conveniently rinsed into colorimeter tubes with a small bulb pipette. The contents of blank Conway units may serve as blanks in the colorimetric method if standards are run in Conway units simultaneously, this is the usual procedure when the interrupted absorption period is used, as in the determination of blood ammonia. When the full absorption period is allowed, it may be more convenient to prepare standards and blanks in the usual fashion and to correct the sample readings for the small and fairly constant value of the blank from the Conway unit.

#### DISCUSSION

The reaction as used by Van Slyke and Hiller and by Borsook was carried out in strongly alkaline solution, the phenol reagent consisting of 25 per cent phenol dissolved in 20 per cent sodium hydroxide. In this

circumstance, when the reaction takes place at or near  $100^{\circ}$ , the color formed is not readily reproducible or suitable for photometric measurement. At lower temperatures, as at  $37^{\circ}$ , recommended by Borsook, the reaction takes some time to go to completion (1 to 2 hours) and the color is less than that produced at  $100^{\circ}$ . However, if the final alkali concentration is made equivalent to the molar strength of the phenol present (pH about 12), the reaction may be carried out at  $100^{\circ}$ , with the production of color about 3 times as intense as that produced by the more strongly alkaline reagent

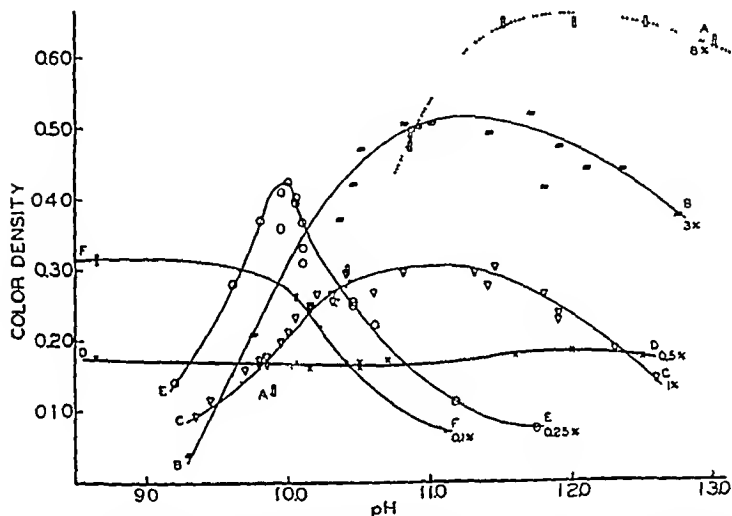


FIG 1 The relationship of pH and of phenol concentration (0.1 to 8 per cent, Curves F to A) to color development in the estimation of ammonia by the phenol-hypochlorite reaction. All samples contained  $10 \gamma$  of ammonia nitrogen and the volume of solution during color development was 10 ml. The concentration of hypochlorite and the measurement of pH are described in the text.

The colored substance produced in this way has a smooth reproducible absorption curve with a maximum at  $625 \text{ m}\mu$ , as determined with a Beckman spectrophotometer at  $10 \text{ m}\mu$  intervals.

The relation of the pH of the reaction and of the final phenol concentration to the density of color produced is shown in Fig 1. The pH was maintained with appropriate buffer mixtures when the phenol concentration was low. The pH was measured with a glass electrode after color development, correction being made for the error due to the presence of sodium ion (4). In the case of the highest concentration of phenol shown in Fig 1 (8 per cent), the pH could not be measured but was calculated from the



composition of the reagent. The optimum pH for concentrations of phenol from 1 to 10 per cent is between 11 and 12, and at this pH the maximum color is obtained with approximately 8 per cent phenol. Lower concentrations, down to 3 per cent, may be used, however, if the amounts of nitrogen present are such as to make a less sensitive reaction desirable. With concentrations of phenol less than 1 per cent, the optimum pH is lower, it is sharply at 10 for 0.25 per cent phenol and between 8 and 9 for 0.1 per cent. (The latter concentration of phenol was used by Hinsburg and Mucke (5), without control of the pH other than initial neutrality of the sample.) Below pH 7, little or no color is produced. It had been expected that at a pH lower than 9 the reaction might be more efficient because of better retention of ammonia, but since only low concentrations of phenol (about 0.1 per cent) could be used here, maximum colors comparable to those produced at higher pH with higher phenol concentrations were not obtained.

The concentration of hypochlorite found satisfactory is nearly that used by Borsook, and Borsook's reagent is recommended. About 20 (15 to 25) mg of available chlorine per ml are required during color development when the phenol concentration is over 1 per cent. With lower concentrations of phenol, the hypochlorite concentration must be carefully balanced with it, in about equimolar proportions with respect to phenol and free chlorine. Such concentrations were used in preparing the curves presented in Fig. 1.

The color production by this reaction is influenced by the presence of certain ions. Iron, chromium, and manganous ions catalyze the reaction,  $Mn^{++}$  is the most effective in enhancing color development, increasing it about 25 per cent, and also increasing its reproducibility. Copper ions tend to inhibit color development.

The density of color produced by the procedure described above, when measured with an Evelyn colorimeter and Filter 620, is a linear function of the concentration of ammonia present. The color is reasonably reproducible but, as in other colorimetric methods, accurate work requires comparison with known standards prepared at the same time. The color is stable for at least an hour after development.

This method of estimating ammonia has not been applied directly to biological fluids, because certain amino acids react to a small extent. Urea does not participate in this reaction. It might therefore be possible under some circumstances to estimate urea by direct determination of ammonia before and after the action of urease. Direct application of the colorimetric method to micro-Kjeldahl digests is not recommended, since traces of peroxide interfere with the reaction, and the control of the final pH offers some difficulties. It would of course be possible to apply the method to

larger samples of ammonia obtained by distillation or aeration procedures other than the Conway technique

#### SUMMARY

The conditions for maximum color development in the reaction of ammonia with phenol and hypochlorite have been investigated and the sensitivity of this test for ammonia has been considerably increased. A simple and reliable colorimetric procedure for estimating very small amounts of ammonia nitrogen has been described, which is suitable for use with the Conway micro diffusion method or other distillation or aeration procedures.

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# INTERFERENCE WITH THE COLORIMETRIC DETERMINATION OF LACTIC ACID (BARKER-SUMMERSON METHOD) BY NITRATE AND NITRITE IONS

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The sensitive and specific method for the determination of lactic acid developed by Barker and Summerson (1) has found wide application since its introduction a few years ago. In this method, lactic acid is first oxidized to acetaldehyde in hot concentrated sulfuric acid. Then the color reaction between acetaldehyde and *p*-hydroxydiphenyl in concentrated sulfuric acid (cold) is utilized for colorimetric measurement. The latter reaction is catalyzed by certain metallic ions,  $\text{Cu}^{++}$  being used in the quantitative method. Although Barker and Summerson state that in the presence of  $\text{Cu}^{++}$  they observed no differences in the results obtained with lots of C.P. sulfuric acid from several sources, the writer and other workers (personal communication) have on occasion found wide variations in the amounts of color obtained in this reaction. With some lots of sulfuric acid, the color obtained is less than the maximum color formed with other acids but is still proportional to the amount of lactic acid present, so that reduction in the sensitivity of the method is the only change. Other lots of acid are quite unusable because of low and irregular color formation.

Recently, Brown (2), in attempting the colorimetric determination of tryptophane by the glyoxylic acid reaction, found a source of interference to be the presence of traces of nitrates or nitrites in the sulfuric acid used. The similarity between this reaction and that used in determining lactic acid suggested that the same difficulty might be encountered in the latter case. This suggestion has been verified experimentally, as shown in Table I. The addition of small amounts of sodium nitrate or nitrite to the samples interfered substantially with color development in the lactic acid reaction.

That differences in the results obtained with different samples of sulfuric acid may be due to the presence of traces of nitrate in the acid is also shown in Table I. Acid A was a satisfactory sample of sulfuric acid, in which the ferrous ammonium sulfate ring test for nitrate was negative. Acid B was unsatisfactory in that the color obtained was only half that obtained with Acid A. According to the manufacturer's label, the maxi-



# NOTE ON THE COLORIMETRIC DETERMINATION OF AMINO NITROGEN

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The color reaction between amino groups and  $\beta$ -naphthoquinone sulfonate, introduced by Folin, was further developed and adapted to photoelectric colorimetry by Frame, Russell, and Wilhelm (1). Since the publication of this paper, experience with the method and the suggestions of other workers have led to certain changes in procedure which appear to increase the stability and uniformity of the color produced in the quantitative reaction.

In order to measure the density of color produced by the reaction of the amino compounds with the  $\beta$ -naphthoquinone sulfonate, it is necessary to bleach the color of the excess quinone reagent with thiosulfate in acid solution. The bleaching procedure of Danielson (2) was used originally and believed to be satisfactory. It has been found that the concentrations of acid and of thiosulfate previously recommended are sufficient to cause noticeable destruction of the colored condensation product as well as of the unchanged reagent. When these reagents are added to the samples before their dilution to final volume as is usually done, the loss of color may be considerable, and since the extent of the loss depends on the time allowed to elapse before dilution, as well as on the concentrations of the reagents, variations in the density of the color finally measured may be introduced here.

The bleaching reagents now used differ from Danielson's in the omission of acetic acid and in the reduction of the strength of the hydrochloric acid and of the sodium thiosulfate used. As found by Danielson, the presence of formaldehyde in a molar concentration slightly less than that of the thiosulfate is required to prevent the precipitation of sulfur in the final acid solution. With the concentrations of acid and thiosulfate recommended below, little destruction of the colored substance occurs, and fading during the first half hour is reduced to negligible proportions.

## *New Reagents—*

Acid formaldehyde 0.30 N HCl containing 0.04 M formaldehyde (3 ml of 40 per cent formaldehyde per liter)

Sodium thiosulfate, 0.05 M

### Procedure

Preparation of the samples, neutralization, and color development are the same as previously recommended, except that a stronger buffer may be preferred (2 per cent borax instead of 1 per cent). After color development and cooling, *dilute the sample to about 13 ml*. Then add 1 ml each of the new acid formaldehyde and thiosulfate solutions, with mixing after each addition, and make the sample to volume (15 ml). It must be noted that if a final volume other than 15 ml is used, the same final concentrations of acid and thiosulfate should be preserved by the addition of proportionate amounts of these reagents. Read the colors 10 to 30 minutes after the addition of the bleaching reagents.

A number of queries have been directed to the author concerning the composition of the blank tubes for use with the photoelectric colorimeter. It was stated in the previous paper that in the determination of blood amino nitrogen it was not necessary to include tungstic acid in the blanks. This of course is true only if the acid and tungstate used to prepare the blood filtrates are not contaminated with chromogenic material. Ammonia, which reacts with the quinone reagent, is frequently such a contaminant, especially of acid solutions. All solutions used in the preparation of samples for analysis by this method should be protected from contamination with ammonia and should be tested occasionally for the presence of chromogenic material by comparison with blanks made with distilled water.

The absorption curves for the colored compounds reported in the previous paper were determined with a Coleman spectrophotometer. It has since been found that the calibration of this instrument was slightly in error. Repetition of some of the curves with a Beckman spectrophotometer has shown the absorption maxima to be closer to 470 m $\mu$  than to 480 m $\mu$  as originally stated.

### SUMMARY

In the colorimetric determination of amino nitrogen, some changes have been suggested in the procedure for bleaching the excess quinone reagent which increase the stability and uniformity of the colors obtained.

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# THE DISTRIBUTION OF WATER AND ELECTROLYTES IN MUSCLE OF DOGS TREATED WITH DESOXYCORTICOSTERONE ACETATE FOLLOWING INJECTIONS OF GLUCOSE AND GLUCOSE-CONTAINING POTASSIUM CHLORIDE\*

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During recent years a number of studies have shown that under certain conditions a replacement of skeletal muscle potassium by sodium can be brought about. Thus, as a striking example, it has been shown by several groups of workers (1-4) that such replacement can be readily induced by repeated injections of desoxycorticosterone acetate, particularly in animals maintained on a high sodium-low potassium diet. It has been our experience (4) that, although under these circumstances the compensatory gain in sodium may be incomplete, one-third or more of the potassium may be lost from the skeletal muscle of dogs injected daily for 2 weeks with 1 mg per kilo of body weight of the substance. Furthermore, despite the marked divergence of the electrolyte pattern from normal, there apparently were no striking changes in the volume of the extracellular and intracellular fluid compartments of the skeletal muscle.

The experiments included in this paper were carried out on dogs in which a replacement of the skeletal muscle potassium by sodium was induced by the administration of desoxycorticosterone acetate. This was done for the following purposes: (1) to observe whether the electrolyte and water exchanges between the extracellular and intracellular compartments of skeletal muscle accompanying an acute loss of extracellular electrolyte, with little change of body water, differ from those observed in normal animals, (2) to compare the electrolyte and phase volume changes of skeletal muscle from normal and experimental animals following the intraperitoneal injection of a glucose solution containing potassium chloride, and (3) to determine whether a change of creatine accompanies the loss of skeletal muscle potassium.

Data from the analyses of heart muscle are also included.

## *Methods*

Adult male dogs were used in this study and were placed on a constant diet (diet suggested by Nilson (5) and supplemented by 15 gm of sodium

\* Aided by a grant from the John and Mary R. Markle Foundation.



chloride and 5 gm of sodium citrate per day) for a period of at least 7 days before the experiments were undertaken. It was customary to withhold food for 18 to 24 hours before collecting the samples for analysis.

The replacement of muscle potassium by sodium was induced by the subcutaneous injection of desoxycorticosterone acetate<sup>1</sup> (1 mg per kilo of body weight daily) for a period of 14 days. Blood was withdrawn under oil by direct puncture of the femoral artery, placed under oil in a special centrifuge tube containing crystalline heparin to prevent clotting, and centrifuged at once for the plasma analyses. Skeletal muscle (lumbar portion, sacrospinalis) was obtained under sodium pentobarbital anesthesia as quickly as possible following the collection of the blood and was sampled for the several analyses in the manner previously described (6). In some experiments the heart was then removed immediately and the left ventricle sampled in the same manner as the skeletal muscle.

After the injection of desoxycorticosterone acetate for a period of 14 days, blood and skeletal muscle of one group of dogs were collected to serve as experimental controls. A second group of dogs was studied following an acute loss of extracellular electrolyte, with little change of body water, produced by the injection of 100 cc per kilo of body weight of 5 per cent glucose solution intraperitoneally and subsequent removal, 4½ hours after the injection, of a volume of fluid equal to that injected. A third group of animals was studied in a similar manner before and following the intraperitoneal injection of a 5 per cent glucose solution containing 50 milliequivalents of potassium chloride per liter.

The following determinations were made on the plasma: water, sodium, potassium, and chloride, and on the muscle: water, chloride, potassium, magnesium, creatine, and total neutral fat. Aside from the determination of creatine, which was carried out by a modification (7, 8) of the method of Rose, Helmer, and Chanutin (9), and of magnesium, which was done essentially as described by Cullen and Wilkins (10), the chemical methods were the same as employed in previous studies (11).

The data collected from the analysis of muscle were expressed on the basis of 100 gm of fat-free solids. The volumes of the extracellular (*F*) and intracellular (*C*) phases of muscle were calculated in the manner outlined by Hastings and Eichelberger (12), which is based on the assumption that all of the chloride is located in the extracellular phase and is present at a concentration equal to that of an ultrafiltrate of plasma. In order to approximate the volume changes produced in the extracellular and intracellular phases of 1 kilo of original muscle, the calculations were made rela-

<sup>1</sup> We are indebted to the Schering Corporation for the generous supply of crystalline desoxycorticosterone acetate in sesame oil (cortate) which was employed for these studies.

tive to the appropriate control series, assuming a constant solid content of the intracellular phase

### Results

The results of the analyses of plasma and skeletal muscle from normal dogs and dogs injected with desoxycorticosterone acetate are summarized in Table I, while the results from the analyses of heart muscle are presented in Table III. In Table II is presented a summary of the calculated skeletal muscle phase values

TABLE I

*Average Electrolytic and Water Content of Plasma and Skeletal Muscle from Normal Dogs and from Dogs Following Administration of Desoxycorticosterone Acetate*

Remarks	No of dogs	Plasma				Muscle per 100 gm fat free solids						
		H <sub>2</sub> O	Na	K	Cl	H <sub>2</sub> O	Cl	Na	(K) <sub>c</sub> *	Mg	Crea tine	
Normal dogs												
		gm per kg	m eq per kg H <sub>2</sub> O	m eq per kg H <sub>2</sub> O	m eq per kg H <sub>2</sub> O	gm	m eq	m eq	m eq	m eq	mg	
Control	25	917.4	156.5	3.2	118.2	320.9	8.0	12.8	37.5	7.9†	1706†	
	σ	6.8	3.1	0.6	4.6	14.9	1.2	1.6	2.8	0.8	137	
Glucose-injected	4	878.9	145.7	2.6	99.1	329.3	4.5	8.4	37.0	8.2	1663	
Before glucose-KCl	4	912.6	156.8	3.3	115.1	315.9	6.9	12.1	38.0	8.1	1710	
After        "	4	891.3	147.3	6.5	116.7	314.0	5.8	8.2	40.6	8.0	1763	
D C A -treated‡												
Control	15	920.2	159.7	2.4	112.6	320.5	7.8	18.3	28.5	7.6§	1690§	
	σ	3.7	3.7	0.5	3.8	16.8	1.1	3.1	4.3	0.6	183	
Glucose-injected	5	893.3	145.2	2.2	93.0	313.6	5.0	11.1	30.5	8.0	1736	
Before glucose-KCl	4	920.7	160.0	2.7	111.1	304.0	6.3	15.7	31.2	7.9	1700	
After        "	4	907.3	157.2	4.3	109.5	305.3	5.0	12.0	32.4	8.1	1688	

\* Corrected for extracellular content

† Eight animals

‡ 1 mg per kilo of body weight of desoxycorticosterone acetate was injected daily for 14 days

§ Five animals

*Muscle Changes Following Injection of Desoxycorticosterone Acetate*—From the data presented in Table I it will be observed that 2 weeks following the administration of desoxycorticosterone acetate there was a marked fall of skeletal muscle potassium which was not completely compensated by a gain of sodium. Thus, the potassium fell from 37.5 milliequivalents,  $\sigma \pm 2.8$  milliequivalents per 100 gm of fat-free solids to 28.5 milliequiva-

lents,  $\sigma \pm 4.3$  miliequivalents, while the sodium increased from 12.8 milliequivalents,  $\sigma \pm 1.6$  miliequivalents, to 18.3 miliequivalents,  $\sigma \pm 3.1$  miliequivalents. On the other hand, neither the water, chloride, magnesium, nor creatinine showed any significant change from normal.

The calculations of the volume changes produced in the extracellular and intracellular phases of 1 kilo of control muscle revealed some irregularity (Table II). Thus, following the repeated injection of desoxycorticosterone acetate the change produced in the bulk of muscle amounted to an

TABLE II

*Average Phase Volume Data of Skeletal Muscle from Normal and Desoxycorticosterone Acetate-Treated Dogs Following Intraperitoneal Injection of Glucose and of Glucose Containing Potassium Chloride*

Remarks		No. of dogs	(H <sub>2</sub> O) <sub>c</sub> *	(F)	$\Delta M$ †	$\Delta C$	$\Delta F$
			gm	gm	gm	gm	gm
Normal dogs	Control	25	721	154			
	$\sigma$		10.0	18			
	Glucose-injected	4	742	101	19	70	-51
	Before glucose-KCl	4	722	139			
D C A -treated‡	After "	4	728	115	-6	18	-24
	Control	15	714	160	-19	-23	4
	$\sigma$		9	17	42	27	21
	Glucose-injected	5	723	126	-2	34	-36
	Before glucose-KCl	4	716	136			
	After "	4	725	108	3	31	-28

\* Per 1000 gm of muscle cell

† Calculations of the changes of the muscle phases of the four normal dogs following glucose injection and of the fifteen control D C A -treated dogs were made relative to the normal control series, assuming a constant solid content of the intracellular phase. Similarly, the changes of the muscle phases of the five D C A treated dogs following glucose injection were calculated relative to the D C A treated control group of animals, while those for the animals injected with glucose plus KCl were calculated relative to the muscle analyzed before injection.

‡ 1 mg per kilo of body weight of desoxycorticosterone acetate was injected daily for 14 days.

average decrease of 19 gm,  $\sigma \pm 42$  gm, per kilo of control muscle, consisting of an average of 23 gm decrease in intracellular phase and an average of 4 gm increase in extracellular phase.

*Muscle Changes Following Acute Loss of Extracellular Electrolyte*—From the average skeletal muscle data for four normal and five desoxycorticosterone acetate-treated dogs presented in Table I, it will be seen that the changes accompanying an acute loss of extracellular electrolyte were similar in the two groups of animals. Essentially, there was only a fall in the amounts of sodium and chloride.

Likewise, the calculations of the volume changes produced in the extracellular and intracellular phases of 1 kilo of control muscle (Table II) revealed similarities in the two groups of animals. In the normal controls the extracellular phase ( $V$ ) of 1 kilo of skeletal muscle amounted to 154 gm,  $\sigma \pm 18$  gm, while that of the desoxycorticosterone acetate-treated dogs amounted to 160 gm,  $\sigma \pm 17$  gm. Following the acute loss of extracellular electrolyte the mean values of ( $V$ ) were found to be 101 and 126 gm, respectively. The changes produced in the bulk of 1 kilo of muscle of normal animals amounted to an average increase of 19 gm per kilo of normal control muscle, consisting of an average of 70 gm increase of the intracellular phase and an average of 51 gm decrease of the extracellular phase. Similarly, the changes in the bulk of 1 kilo of muscle of desoxycorticosterone acetate injected animals amounted to an average decrease of 2 gm per kilo of injected control muscle, consisting of an average of 34 gm increase of the intracellular phase and an average of 36 gm decrease of the extracellular phase. In direction, therefore, the exchanges between the two compartments of skeletal muscle accompanying an acute loss of extracellular electrolyte (sodium) were the same in the two groups of animals.

*Muscle Changes Following Intraperitoneal Injection of Glucose Solution Containing Potassium Chloride*—The skeletal muscle from four normal dogs and four dogs treated with desoxycorticosterone acetate were analyzed before and following the intraperitoneal injection of a 5 per cent glucose solution containing 50 milliequivalents of potassium chloride per liter. From the data presented in Table I it will be observed that in both groups of animals there was a rise in the plasma potassium and a fall of sodium, although the magnitudes of these changes were less in the dogs treated with desoxycorticosterone acetate. On the average, the skeletal muscle analyses revealed a gain of potassium and a decrease of sodium. It will be noted that the increase of the muscle potassium was not very impressive, particularly in the dogs treated with desoxycorticosterone acetate. The smaller gain of potassium in the latter animals was probably related to the lesser rise of plasma potassium.

The calculated changes of the muscle phases (Table II) revealed that the exchanges between the extracellular and intracellular compartments were quite similar in the two groups of animals. Before injection, the extracellular phase ( $V$ ) of 1 kilo of skeletal muscle from normal animals amounted to 139 gm, while that for the desoxycorticosterone acetate treated dogs amounted to 136 gm. Following the injection in the normal animals, the changes in the bulk of 1 kilo of muscle amounted to an average decrease of 6 gm per kilo of original muscle, consisting of an average 18 gm increase of the intracellular phase and an average of 24 gm decrease of the extracellular phase. In the desoxycorticosterone acetate-treated animals, the changes produced in the bulk of 1 kilo of muscle following the injection

amounted to an average increase of 3 gm per kilo of original muscle, consisting of an average of 31 gm increase of the intracellular phase and an average 28 gm decrease of the extracellular phase

*Analysis of Heart Muscle*—The results of the analysis of heart muscle from the animals receiving the intraperitoneal injections are presented in Table III. It will be noted that there were no striking differences between the findings for the normal and desoxycorticosterone acetate treated animals. Following the injection of the solution containing potassium, the average levels for heart muscle potassium were appreciably higher than those found in the control group or in comparable animals injected with

TABLE III

*Average Electrolyte and Water Content and Phase Volume Data of Left Ventricle of Dogs*

Remarks		No of dogs	Muscle per 100 gm fat free solids						(F)	$\Delta M^*$	$\Delta C$	$\Delta F$
			H <sub>2</sub> O	Cl	Na	(K) <sub>c</sub>	Mg	Crea				
			gm	meq	meq	meq	meq	mg	gm	gm	gm	gm
Normal dogs	Control	9	361.5	13.6	18.0	34.7	9.4	1530	246			
	Glucose-injected	4	355.4	8.9	15.2	34.0	7.9	1452	189	-18	43	-61
	Glucose-KCl injected	4	355.0	11.9	15.9	38.8	8.2	1568	216	-18	16	-34
D C A - treated†	Glucose-injected	5	352.7	9.5	17.6	31.5	7.9	1552	216	-24	12	-36
	Glucose-KCl-injected	4	358.3	10.4	17.5	39.2	8.1	1590	198	-13	37	-50

\* Corrected for extracellular content

† Calculations of the muscle phases were made relative to the control series, assuming a constant solid content of the intracellular phase

‡ 1 mg per kilo of body weight of desoxycorticosterone acetate was injected daily for 14 days

the glucose solution alone. There was no evidence of a change in the creatine content. It was interesting to note that the calculated changes of the phase volumes relative to the control were the same in direction as those found with skeletal muscle. That is, the average changes produced in the bulk of heart muscle, per kilo of control muscle, consisted in each instance of an increase in the intracellular phase and a decrease of the extracellular phase.

#### DISCUSSION

It is now generally held that compounds of phosphorus, phosphocreatine, and adenosine triphosphate in particular, play an important rôle in the

energetics of muscular activity. Also there is evidence (13, 14) that these organic phosphoric acid compounds exist as potassium salts, and would thus account for most of the intracellular potassium. Since the repeated injection of desoxycorticosterone acetate, resulting in a fall of skeletal muscle potassium and gain of sodium, may lead to muscular weakness and attacks of paralysis (in dogs) (3, 1) and to cardiac lesions (in rats) (2), it is noteworthy that no change in the creatine content of either skeletal muscle or heart muscle was found in the present study. It is interesting in this connection that Miller and Darrow (1, 15) found that within wide limits the amount of potassium in the muscle cells of rats does not limit its ability to react to electrical stimulation or to exercise.

The repeated injection of desoxycorticosterone acetate resulted in a fall of the skeletal muscle potassium which was not completely compensated for by a gain of sodium. As was pointed out previously (4), the deficit of total cellular electrolyte is accompanied, on the average, by a decrease in the intracellular volume, when compared to the control. Despite the marked divergence of the electrolyte pattern from normal with respect to potassium and sodium, the skeletal muscle was found to respond, in direction, in the same manner as muscle from normal animals to the acute loss of extracellular electrolyte. It would appear, therefore, that the sodium which apparently replaced cellular potassium under these conditions functioned in a similar manner to potassium in controlling the fluid exchange between the two compartments of muscle.

Miller and Darrow (16) and Durlacher and Darrow (17) found that the intracellular sodium of potassium-depleted rats is readily replaced by injected potassium. Further, rats having a deficit of potassium were found to show increased resistance to potassium poisoning and to survive nephrectomy or ureteral ligation longer than rats on a normal diet. In explanation of the above findings the authors concluded that with a depletion of muscle potassium this cation enters the muscle, resulting in a delay in the rise of serum potassium to toxic levels. In the present experiments, the skeletal muscle from normal animals exhibited a small gain of potassium and fall of sodium, following an increase of body potassium by the intraperitoneal injection of a glucose solution containing potassium chloride. Although the magnitude of the changes was not as great, the skeletal muscle from the desoxycorticosterone acetate-treated dogs responded in a similar way. In the latter animals the rise of plasma potassium was not as great as in the controls. The explanation for this is not clear, since both groups of animals were treated in the same way. In contrast to the relatively small increase in skeletal muscle, the analysis of heart muscle following potassium injection revealed an appreciable gain of potassium in both the normal and desoxycorticosterone acetate-treated

animals. In this connection it is interesting to note that Crismon *et al* (18) in experiments with cats found, per unit weight of tissue, the heart took up far more potassium than skeletal muscle, following the intraperitoneal injection of potassium.

#### SUMMARY

A study was made of the creatine content and the electrolyte and water distribution in skeletal muscle of dogs given repeated injections of desoxycorticosterone acetate for a period of 2 weeks, (a) following an acute loss of extracellular electrolyte, with little change of body water, and (b) before and after increase of body potassium by intraperitoneal injection. Analyses of heart muscle were also made in some animals. The various findings may be briefly summarized as follows:

1 The repeated injection of desoxycorticosterone acetate decreased the potassium and increased sodium of skeletal muscle. On the average the sodium increase did not compensate the fall of potassium and the decrease of total electrolyte was associated with some decrease of intracellular volume. No change from normal was found in the creatine content of either skeletal or heart muscle.

2 Despite a marked divergence of the sodium and potassium content from the normal, skeletal muscle from dogs treated with desoxycorticosterone acetate was found to respond, in direction, as normal muscle to an acute loss of extracellular electrolyte with little change of body water.

3 Increase of body potassium by intraperitoneal injection produced a small gain of potassium and fall of sodium in the skeletal muscle of normal animals. Similar changes, although of smaller magnitude, were observed in the skeletal muscle of the dogs treated with desoxycorticosterone acetate. Heart muscle from both groups of dogs gained more potassium than skeletal muscle following potassium injection.

The various findings were discussed briefly.

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# CHEMICAL STUDIES RELATED TO HEMATOPOIETIC ACTIVITY OF BONE MARROW\*

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The red blood cells in man, rats, and other mammalian forms are known to develop primarily, if not exclusively, in the bone marrow. Despite this fact, very little progress has been made in understanding the fundamental changes which occur in the bone marrow during this process. It has been repeatedly demonstrated that copper plays an important rôle in red blood cell formation. Animals anemic as the result of copper deficiency begin forming erythrocytes at an unusually rapid rate shortly after the feeding of small amounts of copper, thus providing an opportunity to study the chemical activity of the bone marrow preceding and during rapid hematopoiesis.

The present study was planned as an analytical survey of various components and fractions of bone marrow during different stages of hematopoiesis. It seemed possible that such data would provide some evidence concerning the precursors and types of reactions involved in hemoglobin and red blood cell formation. The rat was selected as the experimental animal because (a) anemias can be readily developed in this animal, (b) marrow from many individual animals under different conditions can be analyzed, and (c) marrow samples, although small in amount, are more easily separated from bony spicules than in many other species.

A few studies of rat bone marrow during various stages of hematopoiesis have been reported. Schultze (1) found that the cytochrome oxidase activity of bone marrow is markedly reduced in the severely anemic copper-deficient rat. The feeding of copper brought about normal cytochrome oxidase activity within 24 hours. Schultze and Simmons (2), using radioactive copper, demonstrated that copper in small but significant amounts enters the bone marrow during this time interval, thus indicating that the site of the essential function of copper may be within the bone marrow.

Pending resumption of these studies, this paper is presented as a record

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A preliminary report of these studies has appeared (*Federation Proc.*, 1, pt. 2, 124 (1942)).

of the findings for the reference of others who may be interested in similar work

### Methods

*Animals*—Iron- or copper-deficient rats were produced according to the procedure previously described (3). The raw milk,<sup>1</sup> after H<sub>2</sub>S treatment and concentration *in vacuo*, was fortified with enough thiamine chloride to provide a daily intake of at least 20  $\gamma$  of this vitamin. From the time of weaning, the iron-deficient rats received 0.1 mg of Cu<sup>++</sup> plus 0.05 mg of Mn<sup>++</sup> per day and the copper-deficient rats received 0.5 mg of Fe<sup>+++</sup> plus 0.05 mg of Mn<sup>++</sup> per day. Animals on copper therapy developed a copper deficiency and were then given an additional supplement of 0.1 mg of Cu<sup>++</sup> for 5 days.

*Bone Marrow*—The rats were decapitated and the long bones (humeri, femora, and tibiae) removed and split lengthwise with a sharp scalpel, the marrow was then lifted out, care being taken to avoid the inclusion of small splinters of bone. The marrow was suspended in redistilled water in a test-tube and dispersed with a close fitting lucite pestle. (When a glass pestle is used, small particles of glass are found in the suspension and it is impossible, therefore, to determine the total marrow solids satisfactorily.) The suspension was diluted to volume and aliquots were used for the various analytical determinations.

*Determination of Solids*—For total solids, aliquots of the bone marrow suspension containing about 3 to 5 mg of solids were dried *in vacuo* at 60° in an Abderhalden drier and weighed on a micro balance. The water-soluble solids were determined in a similar manner after separation from the water-insoluble solids by centrifuging an aliquot of the suspension for 30 minutes at about 5000 R.P.M. in an angle centrifuge. Duplicate analyses usually differed by less than 1 per cent. It should be noted that a considerable amount of "lipid" was present in the water-soluble fraction, even though these solutions appeared water-clear (Table II, Lines 3, 4).

*Determination of "Lipids"* (a) *Extraction*—The term "lipids" as used here is the sum of the substances extracted by petroleum ether from both alkaline and acid solution after saponification. (Preliminary experiments indicated that saponification permits the extraction of larger amounts of material.)

The samples remaining from the determination of solids were saponified by refluxing for  $\frac{1}{2}$  hour with 0.5 gm of potassium hydroxide and 10 ml of alcohol ether (4 parts of redistilled ethanol and 1 part of redistilled ethyl ether). The solvent was then evaporated in a stream of nitrogen and the residue transferred with redistilled water to the extraction chamber of a

<sup>1</sup> Kindly supplied by Meadow Gold Dairies, Inc., Pittsburgh

liquid liquid continuous extractor, and extracted for 24 hours with petroleum ether which had been washed with concentrated sulfuric acid, followed by water and then redistilled. The alkaline solution in the extraction chamber was acidified with hydrochloric acid and the petroleum ether extraction repeated for another 24 hours. Samples of these two extracts were taken for analysis.

(b) *Oxidation*—When a mixture of fatty acids and non saponifiable lipids is determined by a quantitative wet oxidation procedure, the figures obtained are based upon the use of an empirical factor involving the assumption that the composition of the lipid mixture is identical with that used in determining the factor. Since lipid extracts obtained from tissues probably differ considerably in their composition, it seemed more accurate to express "lipids" in terms of one specific fatty acid (stearic acid) and cholesterol.

An attempt was made to modify the Bloor (4) procedure for use with the small amounts of lipid available. With the Bloor reagent erratic results were obtained and in no case was the recovery of cholesterol satisfactory. Various modifications were tested and the following procedure, involving the measurement of oxidation by determining the chromic ion formed during the reaction,<sup>2</sup> was found to give satisfactory results.

The lipid samples in petroleum ether were transferred to glass-stoppered Pyrex test-tubes, the tubes warmed in a water bath at about 50°, and the solvent completely removed in a stream of nitrogen. Exactly 2.0 ml of the oxidizing mixture (1 part of 0.1 M  $\text{AgIO}_3$  in concentrated  $\text{H}_2\text{SO}_4$  and 7 parts of  $\text{N}/8$   $\text{K}_2\text{Cr}_2\text{O}_7$  in concentrated  $\text{H}_2\text{SO}_4$ ) was measured into each test-tube, the tubes were stoppered and heated in a boiling water bath with occasional mixing for a period of 90 minutes. After cooling, the amounts of chromic ion formed were determined colorimetrically with the open type cell of the Evelyn micro photoelectric colorimeter and a 660  $\mu$  filter.<sup>3</sup> The oxidation of stearic acid and cholesterol was studied for purposes of calibration. The  $K$  values<sup>4</sup> found for stearic acid and cholesterol were 1.395 and 1.504, and were quite constant through a range of 100 to 400  $\gamma$  of sample, at least. The ratio,  $K$  cholesterol to  $K$  stearic acid = 1.079, compared favorably with 1.071 found by Bloor with his oxidative procedure.

<sup>2</sup> Suggested by Dr. W. H. Summerson of Cornell University Medical College.

<sup>3</sup> A titrimetric method was used for some preliminary studies. The same oxidative procedure was used except for a more dilute reagent (4 parts of 0.1 N  $\text{K}_2\text{Cr}_2\text{O}_7$  in concentrated  $\text{H}_2\text{SO}_4$ , 1 part of 0.1 N  $\text{AgIO}_3$  in concentrated  $\text{H}_2\text{SO}_4$ , and 5 parts of concentrated  $\text{H}_2\text{SO}_4$ ). The excess oxidizing agent was determined by thiosulfate titration. 50 to 250  $\gamma$  of palmitic acid or cholesterol could be determined with an accuracy of  $\pm 3$  per cent of the theoretical.

<sup>4</sup>  $K = (100L/C)$ , where  $C$  = micrograms of stearic acid or cholesterol and  $L = (2 - \log G)$ .

The lipid analyses reported in this paper are the sum of the saponifiable material calculated as stearic acid and the non-saponifiable material calculated as cholesterol

*Determination of Nitrogenous Fractions*—The electrometric titration procedure of Borsook and Dubnoff (5) was used for the determination of various nitrogenous fractions of the bone marrow. The apparatus and procedure were modified as noted below, but otherwise were as described by the original authors

*Apparatus*—A glass tube with asbestos fibers sealed into each end and containing saturated KCl was found to be more satisfactory than the agar saturated KCl bridge connection to the calomel electrode. During the titration, the solution was stirred by rotating the titration cup and permitting the electrodes and the tip of the burette to serve as stirrers. This was accomplished by means of a small electric motor with a reduced speed gear attachment turning the cup at about 120 R P M. The microburette assembly used in these determinations is to be described elsewhere

*Total Nitrogen Determination*—The digestions were made in 10 × 75 mm Pyrex test-tubes with a small constriction near the middle and calibrated to a mark made at the point of the constriction (range 1.50 to 1.75 ml). The digestion mixture consisted of a 1:1 dilution of concentrated sulfuric acid with water containing 1 gm of  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  and 0.5 gm of selenious acid per 100 ml of diluted acid

*Procedure*—Aliquots as large as 1.0 ml plus 0.2 ml of digestion mixture were added to digestion tubes and most of the water was evaporated in a drying oven. The tubes were then inserted through small holes in an asbestos board into a sand bath for heating. (The use of micro burners for heating led to occasional loss of sample by spattering, while the sand bath gave very even heating without any loss.) When the solution became clear, the tubes were removed, cooled, 1 drop of saturated potassium persulfate solution added, and the digestion continued for another 15 to 30 minutes. The tubes were removed, cooled, and made up to volume with redistilled water

Samples were transferred from each digestion tube to the outer compartment of distillation cups made from lucite rods of 32 mm diameter and similar to those described by Borsook and Dubnoff (5). Standard acid (about 0.01 N  $\text{H}_2\text{SO}_4$ ) was pipetted into the center compartment and 25 per cent NaOH added to the outer well some distance from the sample. Each cup was covered with a glass plate coated with alkaline glycerol, the alkali and sample were thoroughly mixed by gentle rotation and the cups then placed for 2 hours in a rocker driven at about 40 strokes per minute. The distillation cups were then removed and the acid in the center compartment titrated with 0.03 N NaOH containing about 1 per cent KCl

The difference between this titration and the blanks run simultaneously was the measure of the ammonia in the aliquot

*Ammonia Nitrogen*—Ammonia was determined by Borsook and Dubnoff's procedure, except for the omission of alcohol from the borate reagent (12.4 gm of boric acid plus 100 ml of N NaOH made up to 1 liter and brought to pH 10.3). An accurately measured sample was pipetted into the outer compartment, borate reagent added, and the ammonia determined as in the procedure for total nitrogen. Under these conditions, urea, glycine, asparagine, and nicotinic acid amide did not yield any ammonia, while ammonium sulfate was quantitatively determined.

*"Amide" Nitrogen*—When 25 per cent NaOH reacted with bone marrow fractions under the conditions of the ammonia determination, a much larger amount of ammonia was given off than that produced by borate reagent. It was found that the amide nitrogen of nicotinic acid amide and asparagine are recovered by this procedure, whereas urea and glycine do not react. The nicotinic acid amide reacts quite rapidly, giving quantitative recovery within 3 hours, while asparagine requires approximately 24 hours for complete recovery. The ammonia evolved during a 24 hour interval of contact with 25 per cent NaOH (minus the ammonia from borate reaction) was determined on a number of samples and considered "amide" nitrogen.

*Urea Nitrogen*—Urea was determined by its reaction with urease followed by an ammonia determination with the borate reagent, the difference between the ammonia before and after urease action being the measure of urea present. The sample was placed in the outer compartment of a distillation cup, several drops of a urease suspension added directly to the sample, the cup covered with a glass plate, and the reaction allowed to proceed for 1½ hours, borate added, and the total ammonia evolved measured as in the total nitrogen determination.

*Amino Nitrogen*—In the formal titration for the amino nitrogen of a tissue extract, an arbitrary end-point must be selected (see discussions in (5-7)). The following procedure was adopted for determining amino nitrogen in the bone marrow samples. The tissue extract (pH usually about 7.4) was placed in the center compartment of a distillation cup and titrated to pH 8.00 in an atmosphere of CO<sub>2</sub>-free nitrogen. Formaldehyde solution (U S P formaldehyde diluted 1:1 with water and neutralized to pH 5.0 with NaOH) was added and the sample again titrated to pH 8.00. The second titration, minus a blank titration for the formaldehyde, was assumed to represent the amino nitrogen of the sample.

*Non-Protein Nitrogen*—The proteins were precipitated from the supernatant solution by the addition of sufficient solid trichloroacetic acid to make a 10 per cent solution. After this was mixed well and allowed to

stand for 30 minutes, the protein precipitate was removed by centrifugation. The nitrogen of this solution was determined by the total nitrogen procedure. (During evaporation, this solution should not be heated to more than about 60° because of too rapid decomposition of trichloroacetic acid with loss of sample.) Several non-protein nitrogen samples were also obtained by filtration through a cellophane membrane on an ultrafilter patterned after the one described by Todd (8). The trichloroacetic acid filtrate and ultrafiltrate contained comparable amounts of non-protein nitrogen with slightly lower figures in the ultrafiltrate. The use of the ultrafilter permitted an estimate of the portion of non-protein nitrogen which was present as amino or amide nitrogen.

*Hemoglobin Determination*—Blood hemoglobin was determined by the Evelyn procedure (9) with the micro cell of the photoelectric colorimeter. The  $K$  value used was calculated from hemoglobin determinations by means of the oxygen capacity determined by the manometric procedure of Warburg and compared with the photoelectric colorimeter determination.

The small amounts of hemoglobin present in bone marrow were determined by an adaptation and modification of the methods of Bing and Baker (10) and of McFarlane and Hamilton (11). It is clearly recognized that the procedure is not specific and that other heme compounds may give a similar reaction.

#### *Reagents*—

1 Benzidine 0.5 gm of decolorized and recrystallized benzidine dissolved in 25 ml of 95 per cent ethanol plus 15 ml of glacial acetic acid and diluted to 50 ml with distilled water. (This solution should be kept in the refrigerator and made up frequently.)

2 Hydrogen peroxide About 1.5 per cent, standardized to give a normality of 0.88 to 0.90.

3 20 per cent acetic acid

*Procedure*—Measure 2.0 ml of benzidine reagent into a test-tube and add 1.0 ml of a dilute hemoglobin solution containing 4 to 12  $\gamma$  of hemoglobin. Mix, add 1.0 ml of hydrogen peroxide, mix again, and let stand for 45 minutes. Make up to 20 ml with acetic acid. The per cent transmission ( $T$ ) was determined in the photoelectric colorimeter with a filter having maximum transmission at 520  $m\mu$ . After reduction with ascorbic acid (a few crystals of ascorbic acid added directly to the colorimeter cell), the per cent transmission ( $TR$ ) was again determined. The hemoglobin was calculated from the equation

$$\text{micrograms Hb} = \frac{100L}{K}$$

where  $L = \log (TR) - \log (T)$  and  $K = 4.04$  as standardized against the oxygen capacity of the blood determined manometrically.

## Results

The bone marrow is not a specific organ which can be removed *in toto*, and therefore the samples used for these studies represent a more or less complete recovery of the marrow from six long bones. That the sampling procedure was well standardized is indicated by the fairly constant amount of dry solids obtained from the animals (Table I, Line 5). This fact makes the use of direct comparison of various fractions permissible in interpreting

TABLE I

*Composition of Bone Marrow Solids from Anemic and Normal Rats*

Figures expressed as the mean  $\pm$  standard error of the mean

Description	Normal controls	Iron deficient	Copper deficient	Copper therapy (5 days)
1 No. of rats	9	6	11	8
2 Age, days	78.2 $\pm$ 1.30	70.5 $\pm$ 4.22	74.6 $\pm$ 1.56	74.4 $\pm$ 2.23
3 Weight, gm	94.1 $\pm$ 3.0	94.5 $\pm$ 5.15	66.2 $\pm$ 4.61	65.3 $\pm$ 2.01
4 Hemoglobin, gm per 100 ml blood	13.44 $\pm$ 0.25	4.55 $\pm$ 0.70	2.91 $\pm$ 0.17	7.65 $\pm$ 0.34
Composition of marrow from humeri, femora, and tibiae of each animal				
5 Dry solids, mg	13.00 $\pm$ 1.80	33.65 $\pm$ 1.06	30.61 $\pm$ 1.86	34.08 $\pm$ 1.62
6 Lipids, mg	13.61 $\pm$ 2.48	3.00 $\pm$ 0.35	2.82 $\pm$ 0.37	2.29 $\pm$ 0.21
7 " " per 100 mg dry solids	30.5 $\pm$ 4.7	8.77 $\pm$ 0.90	9.52 $\pm$ 1.50	6.80 $\pm$ 0.63
8 Fat free dry solids, mg	29.36 $\pm$ 1.29	30.65 $\pm$ 0.90	27.79 $\pm$ 1.89	31.79 $\pm$ 1.51
9 Nitrogen, mg	2.97 $\pm$ 0.24	3.93 $\pm$ 0.093	3.60 $\pm$ 0.056	4.03 $\pm$ 0.24
10 " " per 100 mg dry solids	7.11 $\pm$ 0.83	11.69 $\pm$ 0.29	11.70 $\pm$ 0.18	11.80 $\pm$ 0.22
11 Nitrogen, mg per 100 mg fat free dry solids	10.06 $\pm$ 0.54	12.83 $\pm$ 0.20	13.05 $\pm$ 0.28	12.66 $\pm$ 0.19

\* Hb = 3.1  $\pm$  0.21 gm on the day therapy started

the findings. The significantly larger amount of total solids obtained from the normal marrow apparently resulted from the much higher lipid content of this series, for there was little difference between the total fat-free solids from the four groups (Table I, Line 8). The smaller amount of fat-free solids from the marrow of copper-deficient rats compared with that from rats on copper therapy is of doubtful significance. The nitrogen content of normal marrow was significantly lower than that of the animals on the deficient or therapy diets, both in total amount and on a percentage basis (Table I, Lines 9, 10, 11). Although the total nitrogen in copper-deficient



marrow was less than in marrow of iron-deficient rats or those on copper therapy, there was no significant difference upon a percentage basis. The total lipids of the three groups were not significantly different (Table I, Lines 6, 7)

TABLE II

*Water Soluble and Water-Insoluble Fractions of Rat Bone Marrow*

Figures expressed as the mean  $\pm$  standard error of the mean

	Normal controls	Iron deficient	Copper deficient	Copper therapy (5 days)
Water-soluble fraction of marrow from each animal				
1 Solids, mg	17 48 $\pm$ 1 28	19 17 $\pm$ 0 68	19 98 $\pm$ 1 30	20 38 $\pm$ 1 21
2 " " per 100 mg total marrow solids	41 3 $\pm$ 3 0	57 1 $\pm$ 1 92	66 0 $\pm$ 1 34	59 8 $\pm$ 1 35
3 Lipids, mg	3 25 $\pm$ 0 63	1 31 $\pm$ 0 19	1 34 $\pm$ 0 23	1 24 $\pm$ 0 16
4 " " per 100 mg water-soluble solids	18 9 $\pm$ 3 2	7 0 $\pm$ 1 14	6 73 $\pm$ 1 02	6 1 $\pm$ 0 66
5 Fat free solids, mg	14 23 $\pm$ 1 23	17 86 $\pm$ 0 77	18 63 $\pm$ 1 25	19 14 $\pm$ 1 12
6 " " " per 100 mg total fat free marrow solids	48 0 $\pm$ 3 2	56 5 $\pm$ 3 23	67 2 $\pm$ 1 03	60 2 $\pm$ 1 24
7 Nitrogen, mg	1 69 $\pm$ 0 16	2 37 $\pm$ 0 08	2 48 $\pm$ 0 05	2 49 $\pm$ 0 17
8 " " per 100 mg fat-free water sol- uble solids	12 13 $\pm$ 0 80	13 28 $\pm$ 0 23	13 15 $\pm$ 0 31	12 96 $\pm$ 0 15
9 Nitrogen, mg per 100 mg total marrow N	56 4 $\pm$ 1 39	60 3 $\pm$ 1 22	68 8 $\pm$ 1 72	61 7 $\pm$ 1 59
Water insoluble fraction of marrow from each animal				
10 Solids, mg	25 52 $\pm$ 2 07	14 48 $\pm$ 0 94	10 63 $\pm$ 0 72	13 70 $\pm$ 0 66
11 Lipids, "	10 33 $\pm$ 2 00	1 81 $\pm$ 0 22	1 57 $\pm$ 0 30	1 03 $\pm$ 0 10
12 " " per 100 mg water-insoluble solids	37 9 $\pm$ 5 5	12 34 $\pm$ 1 23	14 94 $\pm$ 3 12	7 64 $\pm$ 1 01
13 Fat free solids, mg	15 19 $\pm$ 0 79	12 67 $\pm$ 0 81	9 06 $\pm$ 0 74	12 67 $\pm$ 0 70
14 Nitrogen, mg	1 28 $\pm$ 0 09	1 56 $\pm$ 0 05	1 12 $\pm$ 0 08	1 54 $\pm$ 0 10
15 " " per 100 mg fat-free water in- soluble solids	8 56 $\pm$ 0 62	12 42 $\pm$ 0 37	12 99 $\pm$ 0 98	12 17 $\pm$ 0 42

When the marrow solids were separated arbitrarily into water-soluble and water-insoluble fractions, certain differences in composition were observed. The actual amounts of soluble solids were probably not significantly different, but the percentage of soluble solids of total solids was significantly higher in the copper-deficient marrow than in any other group (Table II, Lines 1, 2). The significantly higher lipid content of the normal

marrow soluble solids is in accord with the significantly lower amount of soluble fat-free solids in this group (Table II, Lines 4, 5). There was no difference in the nitrogen content (actual or percentage) of the soluble solids from the marrow of deficient rats and those on therapy, but the total nitrogen and the percentage nitrogen of the fat-free soluble solids of the normal marrow were lower than any other group (Table II, Lines 7, 8). In parallel with the percentage of total soluble solids of the total solids and the percentage of total fat-free soluble solids of the total fat-free solids, the percentage of soluble nitrogen of the total nitrogen was significantly higher in the copper-deficient marrow than in that from any other group (Table II, Lines 2, 6, 9). From a few analyses, it appeared that the percentage of non protein nitrogen of the total water-soluble nitrogen was not altered significantly in any group (five normal animals average 19.1 per cent, while seven copper-deficient average 20.7 per cent). However, the percentage of non protein nitrogen of the total nitrogen paralleled the percentage of total solids and total nitrogen found in the water-soluble fractions (five normal animals average 8.8 per cent, while seven copper-deficient average 14.3 per cent).

The water-insoluble solids showed more differences in composition than did the soluble solids. The copper-deficient marrow contained significantly fewer insoluble solids than did samples from iron-deficient or copper therapy groups, while the normal marrow contained the largest amount of insoluble solids (Table II, Line 10). These same relationships were found for the fat-free insoluble solids (Table II, Line 13). The total nitrogen in the copper-deficient insoluble solids was lower than in samples from iron-deficient rats or those on copper therapy, but the percentage of nitrogen was not significantly different for these three groups, while the percentage in the normal sample was significantly lower (Table II, Lines 14, 15). Both the total amount and the percentage of lipids were higher in the insoluble solids of normal marrow than in any other group (Table II, Lines 11, 12). The amount and the percentage of lipids in the group on copper therapy were significantly lower than in the copper-deficient group.

Thus, normal marrow samples were found to differ from those from iron-deficient and copper-deficient animals, and rats on copper therapy in their higher lipid content in both the water-soluble and water-insoluble fractions, and also in their lower percentage of nitrogen of the fat-free solids of both these fractions. The water-soluble solids of the three groups did not differ in amount or composition, as shown by lipid and nitrogen analyses. The water-insoluble solids from marrow of the copper-deficient rats were fewer in actual amount and in percentage of the total solids than from that of the iron-deficient rats or rats on copper therapy. Although the percentage of nitrogen of the fat-free insoluble solids was not significantly different in

these three groups, during copper therapy there was a significant decrease in the fat content and an increase in the total insoluble solids and total nitrogen. In copper deficiency (and to a lesser degree in iron deficiency), there is a shift in the proportion between water-soluble and water-insoluble solids in comparison with normal animals. During copper therapy of only 5 days duration, this proportion is significantly altered toward the normal. The insignificant differences in the amounts of water-soluble solids in contrast with the significant differences in the insoluble solids in the four groups of animals suggest that the altered proportion in copper-deficient marrow results from a decreased amount of water-insoluble components rather than from an increased amount of water-soluble material.

The amount of hemoglobin present in bone marrow was quite small (Bone marrow samples from normal, non-deficient, and copper-deficient rats and rats on copper therapy contained, respectively, 71.3, 28.3, 26.6, and 60.2  $\gamma$  of hemoglobin per mg of fat-free solids. Only about 40 per cent of the total hemoglobin was found in the water-soluble fraction.) If all the hemoglobin is formed within the red blood cell before it leaves the bone marrow, then it is possible to approximate the rate of nitrogen turnover involved in these reactions. Assuming that the marrow obtained from these six long bones represents one-half of the total functional marrow of the animal and that no destruction of hemoglobin occurs during the 5 day therapy period, the hemoglobin synthesized during therapy would require at least a complete turnover of the soluble nitrogen every 16 hours.<sup>5</sup> During the first 24 to 48 hours of copper therapy, very little increase in blood hemoglobin can be detected, indicating that an even more rapid synthesis must occur during the periods of most active hematopoiesis.

Amino nitrogen and "amide nitrogen" determinations were made on a number of the water-soluble fractions from each group of animals. There was no indication of any differences in these fractions in the four groups. Of the soluble nitrogen, about 5.5 per cent was present in the form of an "amide" and about 10 per cent as amino nitrogen. Only about 1.4 per cent of the soluble nitrogen was present as urea, with an equal amount present as ammonia. Amino and amide nitrogen fractions were determined in a number of ultrafiltrates. The sum of these two determinations plus the urea and ammonia was in no case more than 30 per cent of the total non-protein nitrogen.

Preliminary studies indicated that a total of about 2 per cent of phosphorus was present in the average copper deficient marrow sample, and not more than one-third of this was in the soluble fraction in contrast with almost 70 per cent of the total nitrogen in the soluble fraction. Total

<sup>5</sup> Unpublished studies of iron metabolism indicate that during rapid hematopoiesis there was the equivalent of complete iron exchange in the bone marrow every 2 hours.

reducing sugar after hydrolysis was determined for a number of samples and found not to exceed 2 per cent of the total dry weight

#### SUMMARY

1 Micromethods for studying various fractions of rat bone marrow have been presented

2 It has been shown that the fat content of bone marrow samples is highly variable, which necessitates the expression of other determinations on a fat-free basis

3 There is a marked shift in the proportion of water-soluble and water-insoluble constituents in the bone marrow of copper-deficient rats in contrast with normal rats. This altered proportion results from a decreased amount of insoluble material rather than an increase in the water-soluble fraction. During a 5 day therapy period, this proportion returns rapidly toward the normal level

4 The hemoglobin content of the marrow of normal rats and of rats recovering rapidly from anemia is relatively small in amount. It follows then that during rapid hematopoiesis the rate at which hemoglobin is synthesized and enters the blood stream is very fast, involving a rapid turnover of nitrogenous material in the bone marrow

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# BIOLOGICAL ACTIVITY OF NATURAL AND SYNTHETIC TOCOPHEROLS\*

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Indications that natural *d*-tocopherols possess more potency than do their respective synthetic *dl* forms developed in our laboratories as a result of repeated bioassays of vitamin E concentrates (natural mixed tocopherols) against synthetic  $\alpha$  tocopherol and *dl*  $\alpha$ -tocopheryl acetate. This contradicted the assumption that natural and synthetic tocopherols are equally potent, which has been widely held ever since Karrer and co-workers (1) synthesized racemic *dl*  $\alpha$ -tocopherol and reported (2) that its biological activity was nearly, if not exactly, equal to that of natural *d*- $\alpha$  tocopherol. Consequently, a plan was evolved whereby our two laboratories, designated as D P I and U R for convenience, could collaborate in testing this matter.

Through the cooperation of Dr. J. G. Baxter and his associates, of the Organic Research Department of Distillation Products, Inc., various natural tocopherols were made available for our use. Pure natural  $\alpha$ -,  $\beta$ -, and  $\gamma$ -tocopherols and certain crystalline esters of *d*- $\alpha$ -tocopherol were assayed against synthetic *dl*- $\alpha$ -,  $\beta$ -, and  $\gamma$ -tocopherols,<sup>1</sup> *dl*- $\alpha$ -tocopheryl acetate, and crystalline *dl*- $\alpha$ -tocopheryl palmitate.<sup>2</sup> The former invariably showed greater potency than the latter compounds.

## EXPERIMENTAL

The diet and bioassay procedure employed in Laboratory D P I was that described by Joffe and Harris (3). In Laboratory U R the technique used was that recently outlined by Mason (4). Consequently, the principal difference was that in the second laboratory the test doses were fed to vitamin E-deficient female rats in equal amounts on each of the first 10 days after conception, whereas in the first laboratory the doses were given in three equal quantities on the 4th, 5th, and 6th days of pregnancy.

\* Communication No. 57 from the Laboratories of Distillation Products, Inc.

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<sup>1</sup> Kindly furnished for these experiments by Dr. J. C. Woodruff of Merck and Company, Inc.

<sup>2</sup> Prepared by Dr. J. G. Baxter and his associates.

Therefore, from the same test dose a slightly better response (lower median fertility dose) might be expected to result in Laboratory U R because of the more efficient use which the animals fed in ten fractional doses might make of it

The spectrographic data were obtained by the use of a medium Hilger quartz spectrograph with a Spekker ultraviolet photometer<sup>3</sup> Persistence

TABLE I  
*Vitamin E Potency of Concentrates of Natural Mixed Tocopherols Bioassayed against Synthetic Tocopherol Standards*

Laboratory	Materials bioassayed*		Bioassay results		
	Un known*	Standard	M f d † of mixed tocopherols in sample	M f d of α tocopherols in standard	α Tocopherol equivalence ‡ of mixed tocopherols in sample
D P I	E-48	International standard	mg	mg	per cent
	E-49	" "	1 33	1 00	75
	E 54	" "	1 33	1 00	75
	E 54	" "	1 00	0 75	75
	E-263	dl-α-Tocopherol (Merck)	1 40	1 10	79
	E 263	" "	1 80	1 25	70
	E 265	" "	1 50	1 00	67
	E 268	" "	1 40	1 00	71
	E 278	" "	1 30	1 00	77
U R	E 285	dl-α Tocopheryl acetate (Hoffmann La Roche)	0 90	0 70	78
	E-136	dl-α Tocopherol (Merck)	0 90	0 72	81
Average and standard deviation					74.8 ± 6

\* Natural mixed tocopherol concentrates (Vegol) ranging in potency from 15 to 48 per cent total tocopherols

† M f d (median fertility dose) represents the quantity of tocopherol necessary to induce positive responses in 50 per cent of a group of vitamin E deficient test rats

$$\ddagger \text{ Per cent equivalence} = \frac{\text{m f d of tocopherol in standard}}{\text{m f d of tocopherols in sample}} \times 100$$

ratios (5) represent  $E_{\max}$  (292 to 300 mμ) to  $E_{\min}$  (253 to 259 mμ) and are considered indicative of the purity of the compounds upon which they are determined

### Results

Table I shows the biological results obtained when various natural tocopherol concentrates were assayed against synthetic tocopherol standards

\* Mr George Wait made the spectrographic measurements

It is evident that the natural mixed tocopherols in the concentrates have exhibited more potency than expected on the basis of their composition (approximately 50 per cent  $\alpha$ -, 50 per cent  $\gamma$ -, and possibly a trace of  $\beta$ -tocopherol). Since natural  $\gamma$  tocopherol is only one twelfth as potent as natural  $\alpha$  tocopherol (3), it would furnish only about 4 per cent  $\alpha$ -tocopherol equivalence in this mixture of the  $\alpha$  and  $\gamma$  forms. Therefore, the  $\alpha$ -tocopherol equivalence of the natural mixed tocopherols would have been expected to be about 54 per cent, 50 per cent from the  $\alpha$ -tocopherol plus 4 per cent from the  $\gamma$  tocopherol present. Actually the average equivalence amounted to approximately 75 per cent (Table I).

TABLE II

*Effect of Non Tocopherol Constituents\* of Tocopherol Concentrate on Biopotency of Mixed  $\alpha$  and  $\gamma$ -Tocopherols*

Group No	Supplement fed		Response to 12 mg total tocopherol	
	Mixed $\alpha$ and $\gamma$ tocopherols	Chromatograph fraction	Litter efficiency	Implant efficiency †
			per cent	per cent
I	Pure compounds		89	85
II	" "	A Skellysolve eluate	88	72
III	" "	B Benzene eluate	89	70
IV	" "	C Ether eluate	56	45
V	Original concentrate		73	75

\* A tocopherol concentrate containing 20 per cent total tocopherols (50 per cent  $\alpha$ - and 50 per cent  $\gamma$ ) was chromatographed on Doucil. Three fractions were obtained by eluting the column successively with Skellysolve, benzene, and ether. These fractions were transferred to olive oil and each was fortified with sufficient amounts of  $\alpha$  and  $\gamma$  tocopherols to bring the total tocopherols of each fraction to a 50 per cent  $\alpha$  and 50 per cent  $\gamma$  tocopherol mixture.

† Implant efficiency (Homrich (7)) =  $\frac{\text{No. of live young}}{\text{No. of total implants}} \times 100$

At first, it was suspected that some non-tocopherol constituent of the vitamin E concentrates (which ranged in potency from 15 to 48 per cent total tocopherols) had exerted an additive or synergistic effect upon the tocopherols present. However, when a typical concentrate was separated by chromatography<sup>2</sup> into four fractions and each of these bioassayed with pure tocopherols, no synergism could be observed (Table II). In fact, one fraction (Fraction C in Table II) seemed to inhibit somewhat the biopotency of the admixed tocopherols.

The apparent anomaly between calculated and actual results might also be explained on the basis of different potencies for the various isomers of tocopherol. Perhaps the natural  $d$  form could be shown to be more active



TABLE III

*Relationship of Potency of Natural  $\alpha$ -Tocopherol Compared with That of Synthetic  $\alpha$ -Tocopherol*

Assay No	Natural tocopherols				Synthetic tocopherols				Ratio of potency of synthetic to natural tocopherols
	No of rats	Amount fed daily	Response (litter efficiency)	M f d * (calculated)	No of rats	Amount fed daily	Response (litter efficiency)	M f d (calculated)	
Laboratory D P I									
1	$\alpha$ -Tocopherol (72 0, )†	10 0 5	0	0 94	$\alpha$ Tocopherol† (74 4, 3 0)†	8 0 75	13	1 32	1 1 4
		10 0 75	10			7 1 00	29		
		9 1 00	67			7 1 50	100		
		12 1 25	92						
2	$\alpha$ -Tocopheryl succinate (38 5, 5 7)	16 0 60	13	1 0	$\alpha$ Tocopheryl acetate‡ (36 8, 3 0)	11 1 0	18	1 29	1 1 3
		13 0 85	31			10 1 5	70		
		11 1 2	64						
		14 1 7	100						
3	$\alpha$ -Tocopherol (79 7, 8 9)	10 0 6	20	0 79	$\alpha$ -Tocopherol† (77 5, 3 0)	6 0 6	0	1 0	1 1 3
		9 1 0	78			12 1 0	50		
4	$\alpha$ -Tocopherol (73 0, 6 0)	9 0 6	33		" "	8 1 0	38		1 1 7
5	$\alpha$ -Tocopheryl succinate (38 5, 5 7)	18 0 75	38		" "	10 1 00	50		1 1 4
6	" "	10 0 6	50	0 6	$\alpha$ -Tocopheryl acetate   (30 6, 1 8)	9 0 6	0	0 9	1 1 5
		12 0 8	92			9 0 8	33		
		10 1 0	100			9 1 0	67		
7	$\alpha$ -Tocopheryl acetate (crystalline) (41 2, 4 0)	7 0 4	14	0 47	" "	10 0 4	10	0 70	1 1 5
		6 0 55	83			9 0 55	0		
		7 0 70	100			8 0 70	50		
		6 0 85	100			10 0 85	90		
		6 1 00	100			10 1 00	90		
8	" "	8 0 25	7	0 40	$\alpha$ Tocopheryl acetate   (36 0, 3 0)	9 0 50	5	0 91	1 2 3
		8 0 37	36			9 0 75	29		
		9 0 50	86			12 1 00	59		
		9 0 75	96			6 1 25	100		
		9 1 00	100						
9	$\alpha$ Tocopheryl palmitate (crystalline) (26 8, 2 5)	8 0 25	13	0 42	$\alpha$ -Tocopheryl palmitate (crystalline) (30 9, 1 9)	9 0 5	11	0 77	1 1 8
		8 0 37	38			9 0 75	44		
		9 0 5	89			12 1 00	42		
		9 1 00	100			3 1 25	100		
10	$\alpha$ Tocopheryl palmitate (crystalline) (26 8, 5 7)	8 0 5	87		$\alpha$ -Tocopheryl palmitate (crystalline) (30 9, 7 8)	8 0 5	25		

TABLE III—Concluded

Assay No	Natural tocopherols					Synthetic tocopherols					Ratio of potency of synthetic to natural tocopherols
	No of rats	Amount fed daily	Response (litter efficiency)	M f d (calculated)	No of rats	Amount fed daily	Response (litter efficiency)	M f d • (calculated)			
Laboratory U R											
1	$\alpha$ Tocopherol (79 7, 8 9)	10 0	375	0	0 62	$\alpha$ Tocopherol† (71 4, 5 8)	8 0	5	0	0 72	1 1 2
		8 0	50	0			11 0	583	9		
		11 0	583	45			18 0	75	39		
		10 0	675	50			8 0	825	88		
		11 0	75	91			11 1	0	100		
		10 1	00	100							
	$\alpha$ -Tocopheryl succinate (38 5, 5 7)	9 0	5	11	0 58						
		11 0	583	36							
		22 0	675	96							
		9 0	75	100							
		8 1	00	100							
Average (approximate)											1 1 5

\* See Table I for the significance of m f d

† The figures in parentheses refer to the value of  $D_{1\text{cm}}^{1\%}$  (285 to 295  $m\mu$ ) and to the persistence ratio of the compound used, respectively

‡ Merck

§ Hoffmann La Roche

|| International standard

biologically than the *dl* racemic mixture, as are certain other vitamins and amino acids. That this is indeed true is indicated in Tables III and IV. A more direct procedure, of course, would be to isolate the synthetic isomers (6) of the tocopherols and assay them biologically against the natural *d* form. This isolation is being attempted, but in the meanwhile it is possible to conclude from the results in Table I that natural *d*- $\alpha$ -tocopherol is over 40 per cent more potent than synthetic *dl*- $\alpha$ -tocopherol, *z e*,

$$\frac{(\text{Determined equivalence}) - (\text{calculated equivalence})}{\% d \alpha \text{ tocopherol in total mixed tocopherols}} \text{ or } \frac{74.8 - 54.0}{50.0} = 41.6\%$$

Both the determined and calculated equivalences are expressed as per cent synthetic  $\alpha$ -tocopherol equivalence. This calculation is, of course, based on the assumption that the  $\alpha$ - to  $\gamma$ -tocopherol ratio in the concentrate is exactly unity.

Likewise, natural *d*- $\beta$ -tocopherol was assayed against synthetic *dl*  $\beta$ -tocopherol with the results shown in Table IV. The *d*- $\beta$ -tocopherol is apparently twice as potent as *dl*- $\beta$ -tocopherol with median fertility dose values of 1.5 and 3.0 mg, respectively.

In attempting to compare the relative bioactivity of natural and synthetic  $\gamma$ -tocopherol, a median fertility dose of 9.0 mg was determined for the *d* form, but doses of the *dl* form up to 100.0 mg were without vitamin E activity (Table IV). However, in Laboratory U R a 50 per cent response for synthetic *dl*- $\gamma$ -tocopherol was obtained in one assay at a level of 5.0 mg. This point needs further investigation as soon as more synthetic  $\gamma$ -tocopherol is available for feeding purposes.

#### SUMMARY

Natural  $\alpha$ -,  $\beta$ -, and  $\gamma$ -tocopherols, in pure form and as crystalline esters, have been assayed biologically against the corresponding synthetic *dl*-tocopherols and esters.

Natural  $\alpha$ -tocopherol proved to be approximately 50 per cent more potent than synthetic *dl*- $\alpha$ -tocopherol. Natural  $\beta$ -tocopherol showed about 100 per cent more activity than the synthetic *dl*  $\beta$ -tocopherol. However, the activity relationship of natural  $\gamma$ -tocopherol and synthetic  $\gamma$ -tocopherol was difficult to establish. In one laboratory, synthetic  $\gamma$ -tocopherol was found to be one half as potent as the natural form, whereas in the other cooperating laboratory, it was less than one-tenth as active as natural  $\gamma$ -tocopherol; 100 mg doses consistently gave negative responses.

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# THE CHEMICAL ESTIMATION OF TOCOPHEROLS IN BLOOD PLASMA\*

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The study of vitamin E in nutrition has been hampered for want of a simple method of accurately measuring tocopherols in complex mixtures, especially in blood plasma. Emmerie and Engel have applied their well known method (1) to the measurement of tocopherols in the serum of rats, and a similar technique has been used by Wechsler, Mayer, and Sobotka (2, 3) for human serum. However, their procedures are time consuming and, since they involve a chromatographic removal of the interfering vitamin A and carotenoids, are not entirely suitable for routine use. Scudi and Buhs (4) have used a modification of the vitamin K method for measuring tocopherol in dog plasma, however, this technique, though apparently specific for tocopherols, is too intricate for routine use.

Accordingly, we have sought to devise a method which combines a rapid, relatively simple technique with reasonable specificity for tocopherols and freedom from interference by carotenoids. The procedure here described takes about 80 minutes and involves lipid extraction, mild hydrogenation to obviate interference due to vitamin A, carotenoids, and other unsaturated substances, and use of the Emmerie and Engel reagents for measurement of the tocopherols.

## EXPERIMENTAL

### Reagents—

- 1 Absolute ethyl alcohol
- 2 Purified Skellysolve B<sup>1</sup>
- 3  $\alpha, \alpha'$ -Bipyridine, 0.25 gm in 50 ml of absolute alcohol. This should be kept in a dark bottle.
- 4 Ferric chloride hexahydrate, 0.10 gm in 50 ml of absolute alcohol. This also should be kept in a dark bottle.
- 5 Palladized calcium carbonate (hydrogenation catalyst)<sup>2</sup>

\* Communication No. 63 from the Laboratories of Distillation Products, Inc.

<sup>1</sup> Purified according to the directions of Hines and Mattill (5) by shaking three times with concentrated sulfuric acid, and washing with water, dilute sodium hydroxide, and water, drying over anhydrous calcium chloride, and distilling.

<sup>2</sup> Prepared according to directions given in Gattermann and Wieland (6) as follows: "Suspend 50 g of well washed calcium carbonate which has been precipitated while hot in about 200 cc of water and mix the suspension by shaking and gentle

### Procedure

5 ml of plasma from ovalated blood are placed in a 50 ml glass stoppered cylinder or centrifuge tube. 5 ml of absolute alcohol are added to this and gently mixed. Then exactly 12 ml of purified Skellysolve B are added. The stopper is greased with starch-glycerol jelly and inserted securely in the cylinder or tube. This is shaken continuously for 10 minutes, after which it is let stand or centrifuged until the layers separate.

10 ml of the Skellysolve B layer are then transferred to a 25 ml Erlenmeyer flask and evaporated just to dryness under nitrogen on a water or steam bath. While the residue is still warm, it is taken up in exactly 10 ml of absolute alcohol. The flask is corked and rotated several times to insure solution of the residue. This step is of critical importance, since low values will result if complete solution is not obtained.

The alcohol solution is decanted into a hydrogenation bottle and 0.1 to 0.2 gm of catalyst added. The bottle is evacuated and filled with hydrogen four times and then hydrogenated at 35 pounds pressure for 20 minutes, with agitation, at room temperature. The instrument we have used is the Parr catalytic hydrogenation apparatus, model CA, Parr Instrument Company, Moline, Illinois. After hydrogenation the solution is poured as completely as possible into a centrifuge tube and centrifuged until the supernatant fluid is clear.

A convenient amount (8 ml if possible) of the supernatant fluid is pipetted into an Evelyn photometer tube and, if necessary, absolute alcohol is added to make a total volume of 8 ml.

The Evelyn colorimeter is adjusted to the correct "center setting" and then to the unknown is added 1 ml each of  $\alpha, \alpha'$ -bipyridine and ferric chloride reagents, the latter from a rapid delivery pipette (less than 5 seconds). The tube is shaken to mix the contents. The intensity of color developed is read at exactly 15 seconds after the final portion of ferric chloride is added. The tocopherol content of the sample in the tube is found from a calibration curve prepared with samples of pure natural  $\alpha$ -tocopherol in absolute ethyl alcohol, with amounts of 25 to 100  $\gamma$  of tocopherol in 8 ml of alcohol.<sup>3</sup>

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warming with a solution of 1 g of palladium chloride. When the liquid over the calcium carbonate is completely decolorised, pour off, wash the solid a few times with distilled water, and then filter at the pump. Wash the material on the funnel until the filtrate is free from chlorine ions, dry in a vacuum desiccator and store in a well-stoppered reagent bottle. The adsorbed palladium hydroxide is reduced during the determination." We have also used a sample of "5 per cent Pd-CaCO<sub>3</sub> catalyst" purchased from Baker and Company, Inc., Newark, New Jersey, with satisfactory results.

$$^3 \text{ Sample Calculation—Mg per cent tocopherols} = \frac{100}{\text{ml plasma}} \times \frac{\text{total ml SSB}}{\text{aliquot SSB}} \times$$

To find the "center setting," a blank is prepared as follows. To 8 ml of absolute alcohol in a photometer tube 1 ml of  $\alpha, \alpha'$ -bipyridine reagent and 1 ml of ferric chloride reagent are added. With the tube in the instrument, and with Filter 520, the galvanometer is set so that it reads 100 exactly 15 seconds after the last drop of ferric chloride is added. The tube is then removed from the colorimeter and the galvanometer reading noted. This reading, the "center setting," must be determined each day and with each change of reagents. Should the "center setting" with fresh reagents be off the scale, it may be brought to a reading below 100 by slight manual adjustment of the colorimeter light bulb. Older reagents which do not give a blank within a few points of that given originally should be replaced with fresh reagents.

#### DISCUSSION

The extraction step resembles that of Kimble (7) for vitamin A and it was adopted with some confidence because Yudkin (8) has shown it to be as efficient for vitamin A as prior saponification and extraction with ether. We have found in partition experiments that  $\alpha$ -tocopherol is taken up completely by the Skellysolve B layer when shaken with the proportions of alcohol, water, and Skellysolve B used in the analytical procedure.

The new hydrogenation step is believed to obviate interference by carotenoids and vitamin A, since alcoholic solutions of crystalline vitamin A and carotene of appropriate concentrations give no reduction of the Emmerie and Engel reagent after hydrogenation as described.

A 15 second time interval for the Emmerie and Engel reading is used and it was found that under the conditions described for the reaction, solutions of pure  $\alpha$ -tocopherol show an  $L$  value directly proportional to concentration. This proportionality has been reproduced by several different laboratories and technicians. Since blood lipid extracts, even after hydrogenation, give color which shows a greater increase with time than do pure

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$$\frac{\text{total ml EtOH}}{\text{aliquot EtOH}} \times \text{mg tocopherols in aliquot EtOH} = \frac{100}{\text{ml plasma}} \times \frac{12}{10} \times \frac{10}{10}$$

$$\frac{10}{\text{aliquot EtOH used}} \times \text{mg tocopherols (in aliquot EtOH)}$$
 If 5 ml of plasma are taken, 8 ml of the alcohol solution used, and 0.040 mg of tocopherols found, then
 
$$\text{mg per cent tocopherols} = \frac{100}{5} \times \frac{12}{10} \times \frac{10}{8} \times 0.040 = 1.20$$
 A formula can also be used for the calculation of mg per cent of tocopherols in plasma, provided constant amounts of plasma and aliquots of the hydrogenated alcohol solution are used, e.g., 5 and 8 ml, respectively.  $C = K \times L$  where  $C$  = concentration of plasma tocopherol expressed in mg per cent,  $K$  = a constant, and  $L$  = the customary  $(2 - \log G)$ . With a composite calibration curve constructed from repeated standardizations of pure natural  $\alpha$ -tocopherol, a value of 4.27 was found for  $K$  in the above formula.

tocopherol solutions, it would seem that readings made at longer time intervals measure extraneous reducing materials in addition to tocopherols

The Emmerie and Engel reagent is not specific for tocopherols and values obtained with its use cannot *a priori* be regarded as a direct measure of vitamin E. Accordingly, we have checked the chemical assay with biological determinations. Freshly drawn beef blood, obtained through the courtesy of the Rochester Independent Packing Company, was centrifuged, and the serum frozen and then dehydrated under a vacuum by Mr George Kuipers. A residue which weighed 8.5 per cent of the original resulted, and this was fed to rats according to standard bioassay procedure (9). Dr J. L. Jensen and Miss Marion Ludwig conducted the bioassays.

TABLE I  
*Replicate Analyses of Blood for Tocopherols*

Sample	Sample No	Amount	Tocopherol levels	
				Average
		ml	mg per cent	mg per cent
Human plasma	1a	5	1.20	
	1b	3	1.20	1.20
" " *	2a	5	1.82	
	2b	3	2.00	1.91
" "	3a	4	1.11	
	3b	4	1.29	
	3c	4	1.16	1.19
	4a	6	0.34	
Beef serum†	4b	6	0.37	0.36

\* Subject received a daily dose of 25 mg of natural mixed tocopherols for 2 weeks.  
† 8.5 per cent reconstituted, dehydrated serum.

After a preliminary multilevel test to determine approximate potency, an extensive bioassay at 5, 10, and 15 gm feeding levels resulted in a median fertility dose of 10.5 gm. The reference standard used in this assay was pure natural  $\alpha$ -tocopheryl acetate which showed a median fertility dose of 0.50 mg of tocopherol. Consequently, it was calculated that the reconstituted serum contained 0.40 mg per cent of tocopherol.

An 8.5 per cent solution in distilled water of the dehydrated serum was assayed chemically by the above procedure. Eight analyses on aliquots of five solutions indicate an average of 0.36 mg per cent of tocopherols, in good agreement with the value by bioassay.

#### *Application of Method*

As mentioned above, a series of eight analyses on various solutions of reconstituted dried beef serum shows an average of 0.36 mg per cent of

tocopherols. Extreme values were 0.32 and 0.41 mg per cent of tocopherols. Replicate analyses of blood samples show good agreement, as listed in Table I.

TABLE II  
*Recovery of  $\alpha$  Tocopherol Added to Reconstituted Dried Beef Serum*

Tocopherol content of sample (a)	Tocopherol added (b)	Total tocopherol found (c)	Recovery $\frac{(c) - (a)}{(b)} \times 100$
mg per cent	mg per cent	mg per cent	per cent
0.36	0.48	0.84	100.0
0.36	0.96	1.27	94.8
0.36	2.00	2.49	106.5
0.36	4.00	4.30	98.5
Average recovery			100.0

TABLE III  
*Range of Plasma Tocopherols in Normal Individuals*

Subject No	Age	Sex	Vitamin E in 100 ml plasma
	yr		mg
1	17	F	0.90
2	16	"	0.93
3	25	M	1.02
4	18	F	1.03
5	20	M	1.07
6	25	"	1.19
7	26	F	1.20
8	18	"	1.23
9	48	"	1.24
10	19	"	1.27
11	36	"	1.34
12	34	M	1.58
13	24	F	1.59
Average			1.20

Recovery experiments were made in which  $\alpha$ -tocopherol dissolved in absolute alcohol was added to the reconstituted serum solution before extraction. The average recovery was 100 per cent, as shown in Table II.

A series of blood plasma samples (obtained from the Eastman Kodak Medical Department through the cooperation of Dr J. H. Sterner) from normal subjects was assayed with the results given in Table III. The



concentration of tocopherols in normal blood plasma appears to vary from 0.9 to 1.6 mg per cent, 1.2 mg per cent being average. Wechsler *et al* (2, 3) have reported somewhat lower values suggesting the normal range to be 0.6 to 1.0 mg per cent in serum.

The rate of appearance of tocopherols in the blood of a normal subject following ingestion of 1500 mg of natural mixed tocopherols was determined. Samples withdrawn at intervals of 0, 2, 4, 6, 8, 24, and 48 hours were assayed. During this period, the subject ate light meals composed of low fat foods. Fig 1 shows the results. From a fasting level of 1.34 mg

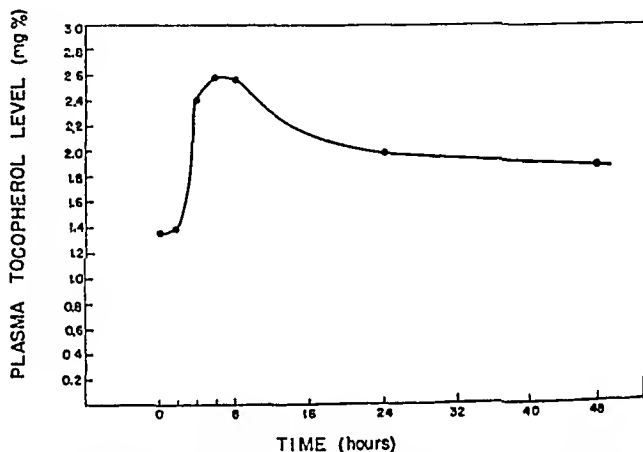


FIG 1 Tocopherol tolerance curve for a normal subject after ingestion of 1500 mg of natural mixed tocopherols

per cent, the plasma tocopherol level rose to a maximum of 2.60 mg per cent at 6 hours and then fell to 2.04 mg per cent at 24 hours and 1.92 at 48 hours (Fig 1).

Ideas and technical assistance were contributed by Mr Edgar Shantz, Dr Ellenmae Viergiver, Mr John Cobler, and Mr Hugh Risley while this method was being developed.

#### SUMMARY

1 A chemical method for determining total tocopherols in blood plasma is described. Hydrogenation is used to prevent interference by carotenoids and vitamin A. Tocopherols are measured by the Emmerie and Engel reaction.

2 The chemical method has been checked by bioassay of dehydrated beef serum.

3 Values for a small series of human normal plasma tocopherol levels were found to be between 0.9 and 1.6 mg per cent with an average of 1.20 mg per cent

4 The rate of appearance of tocopherols in the blood of a normal subject following ingestion of a large dose is shown

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# THE TRYPTOPHANE CONTENT OF FEEDSTUFF PROTEINS

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Eckert (1) reviewed the existing methods for the colorimetric determination of tryptophane and described a new micro colorimetric method for its determination. Since there is considerable discrepancy in the tryptophane content of feedstuffs as determined by the older methods (2-20), Eckert's method has been adapted to determine tryptophane in feedstuffs. Analyses of several feedstuffs have been made in an attempt to obtain more reliable data concerning the tryptophane content of these feedstuffs.

## Method

A 1 to 2 gm sample was autoclaved with 3.5 gm of barium hydroxide and 25 cc of water for 5 hours at 15 pounds pressure per sq in. The hydrolysate was then transferred to a 100 cc volumetric flask, neutralized with sulfuric acid, diluted to volume with water, and filtered.

Eckert's method (1) was used to determine the tryptophane in 1 and 2 cc aliquots of the filtered hydrolysate, and the micro-Kjeldahl method was used to determine the total nitrogen in a 1 cc aliquot. The tryptophane content of the feedstuff protein was calculated on the assumption that the total nitrogen of the filtrate of the feedstuff hydrolysate, when multiplied by the conventional factor 6.25, represents with sufficient accuracy the protein content of the feedstuff. The results are given in Table I and in each case are the averages of two or more determinations. Comparable tryptophane values of other investigators are also shown.

## DISCUSSION

Although many investigators have determined the tryptophane content of casein, the results show wide discrepancies. Jones *et al* (6), Milone and Everitt (9) and Sullivan *et al* (13) obtained values of approximately 2.4 per cent, or about twice that found by Eckert's method. The values given by Block and Bolling (2), Folin and Ciocalteu (3), May and Rose (8), and Folin and Looney (12) are about 1.5 per cent, or 25 per cent greater than those found for casein in the present investigation. On the other hand, the results of Holdiday (5), Luscher (7), and Shaw and McFarlane

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(10, 11) are in excellent agreement with the present value and also with that obtained by Greene and Black (4) by their microbiological method. Since this work was completed, Brown (21) found casein to contain 1.02 per cent tryptophane by using Ecker's method, but he used sodium hydroxide

TABLE I  
*Tryptophane Content of Feedstuff Proteins*

Source of protein	Tryptophane per cent of crude protein	
	Author's data	Data from literature
Casein	1.2	1.5 (2), 1.02 (21), 1.4 (3), 1.15 (4),* 0.85-1.26 (5), 2.2 (6), 1.1-1.39 (7), 1.5 (8), 2.44 (9), 1.16 (10), 1.2 (11), 1.54 (12), 2.4 (13)
Alfalfa meal	2.3	2.4 (14)
Wheat	0.9	1.0 (2), 1.0 (15), 0.9 (16)
" bran	1.0	
Barley	0.8	
Corn (yellow)	0.5	0.6 (2), 0.7 (15), 0.5 (17)†
" gluten meal	0.4	
Soy bean oil " (solvent process)	1.0	1.5 (2), 1.5 (15)
" " " (expeller " )	0.9	
Cottonseed meal	0.9	1.3 (2)
Linseed meal	1.2	1.9 (2)
Sesame "	1.2	
Peanut "	0.7	1.0 (2)
Hemp seed meal	1.0	
Yeast	1.0	1.4 (2), 1.4 (15)
Dried buttermilk	0.9	
Sardine meal	1.1	1.5 (18)†
Tuna meal	1.1	
Menhaden stick	0.6	
Meat scrap	0.7	0.7 (15)
Liver meal	1.0	
Blood "	1.3	1.5 (2), 1.0 (19)
Red cell, blood fraction	1.6	
Fibrin (beef)	3.5	3.5 (20), 3.7 (15)

\* Calculated to moisture-free basis

† Calculated to 16 per cent nitrogen

for hydrolysis rather than barium hydroxide. Sodium hydroxide was tried in the early phases of this work and was found to give approximately 1.0 per cent tryptophane in casein, but this reagent was discarded in favor of barium hydroxide because many erratic results were obtained.

The values in Table I for soy bean oil meal, cottonseed meal, linseed

meal, peanut meal, yeast, and sardine meal are approximately 70 per cent of the respective values reported in the literature (2, 15, 18). The results for alfalfa, wheat, corn, meat scrap, and blood meal are approximately the same as those given by other investigators (14, 2, 15-17, 19). A tryptophane content of 3.5 per cent for bovine fibrin compares favorably with the values of 3.5 per cent reported by Brand and Kassell (20), and 3.7 per cent by Block and Bolling (15).

## SUMMARY

The tryptophane content of various feedstuff proteins was determined by Eckert's method after hydrolysis of the feedstuff in the autoclave by barium hydroxide.

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flask and the trichloroacetic acid precipitate is washed once with about 10 ml of 4 per cent trichloroacetic acid. To obtain consistent results, only one washing is desirable. The volumetric flask is made up to volume and the contents filtered so as to remove particles which are not centrifuged down. All operations should be carried out at 0°. Kay (7) has found that when trichloroacetic acid precipitates from tissues are left in contact with the acid there occurs a progressive increase in acid-soluble phosphorus and nitrogen. We have confirmed Kay's observations and have also found that the amount of acid-soluble phosphate extracted is dependent on the number of washings with trichloroacetic acid.

The nature of the increased phosphate, which results from hydrolysis by trichloroacetic acid, will be discussed later in this paper.

*Fractionation of Acid-Soluble Phosphates with Ba(OH)<sub>2</sub>*<sup>1</sup>—The method of separation of the acid-soluble phosphates of liver is based on the procedure of Eggleton and Eggleton (8) for separating muscle phosphates. A barium precipitate is obtained by adding crystalline Ba(OH)<sub>2</sub> to the turning point of phenolphthalein in the manner described by Cori and Cori (9). The neutralization of the trichloroacetic acid extract is carried out at 0°.

Whenever the glycogen content of liver exceeds 2.5 per cent, a distinct opalescence of the extract occurs. Glycogen is known to hold some of the barium-insoluble phosphates in colloidal suspension. Therefore in order to obtain accurate results, glycogen must be removed before any fractionation is possible. Glycogen may be removed by adsorption on mercuric sulfide as described by Flock, Bollman, and Mann (4). It should be pointed out that removal of glycogen by adsorption is necessary only when the extract is opalescent. The glycogen effect may, in certain instances, be overcome by dilution of the opalescent extracts.

The precipitate resulting from the treatment with Ba(OH)<sub>2</sub> is centrifuged down, dissolved in a minimum quantity of 4 per cent trichloroacetic acid, and again precipitated by neutralization with Ba(OH)<sub>2</sub>. After centrifugation, the precipitate is dissolved in a few ml of dilute HCl and made up to volume (25 ml) with distilled water. This will be called the fraction of insoluble barium salts.

The supernatants from the barium precipitate are combined and then 0.6 ml of 1 N acetic acid and 2.5 ml of 20 per cent mercuric acetate are added for each 25 ml of solution. A flocculent precipitate results which is centrifuged out after standing in the ice box overnight. The precipitate is dissolved in dilute acid (4 per cent trichloroacetic) and H<sub>2</sub>S bubbled in. The resulting HgS precipitate is centrifuged down and the supernatant

<sup>1</sup> It was our usual procedure to use half of the trichloroacetic acid extract for barium fractionation.

aerated After aeration, the contents are made up to volume (10 or 25 ml) This fraction will be referred to as the Hg precipitate (fraction of soluble barium salts)

The supernatant from the Hg precipitate is concentrated on a steam bath to 15 to 20 ml, and then treated with 2 ml of a saturated neutral lead acetate solution The small resulting precipitate is centrifuged off after first standing in the ice box for 24 hours  $\text{H}_2\text{SO}_4$  is then added to remove the Pb The filtrate from the  $\text{PbSO}_4$  precipitate is made up to volume and will be referred to as the Pb precipitate (fraction of soluble barium salts)

The supernatant from the Pb precipitate is transferred to a 250 ml centrifuge bottle, 3 volumes of alcohol are added and the mixture is kept in the refrigerator overnight The resulting precipitate contains most of the barium-soluble phosphates The precipitate is treated with 4 per cent trichloroacetic acid and  $\text{H}_2\text{S}$  is bubbled in,  $\text{H}_2\text{SO}_4$  is also then added so as to remove any barium which might be present The supernatant is aerated and made up to volume This fraction will be called the alcohol precipitate (fraction of soluble barium salts) Only traces of phosphate are found in the alcohol supernatant and therefore it is discarded

The scheme for separating the acid-soluble phosphate fractions is represented diagrammatically

### *Composition of Acid-Soluble Phosphate Fractions of Liver*

#### *Fraction of Insoluble Barium Salts*

A number of phosphate compounds are known to be barium-insoluble They are inorganic phosphate, adenosine diphosphate, adenosine triphosphate,<sup>2</sup> the phosphoglyceric acids, and fructose diphosphate (Harden and Young ester) These different compounds are determined according to the following scheme

One aliquot of the fraction of insoluble barium salts is used for total P determination by digestion with 10 N  $\text{H}_2\text{SO}_4$  and  $\text{H}_2\text{O}_2$  After digestion, the liberated inorganic phosphate is determined colorimetrically according to the Fiske-Subbarow procedure (10)

*Inorganic Phosphate*—The inorganic phosphate is determined by the Fiske-Subbarow procedure directly on an aliquot of the fraction of insoluble barium salts The color readings were made on the Klett-Summerson photoelectric colorimeter

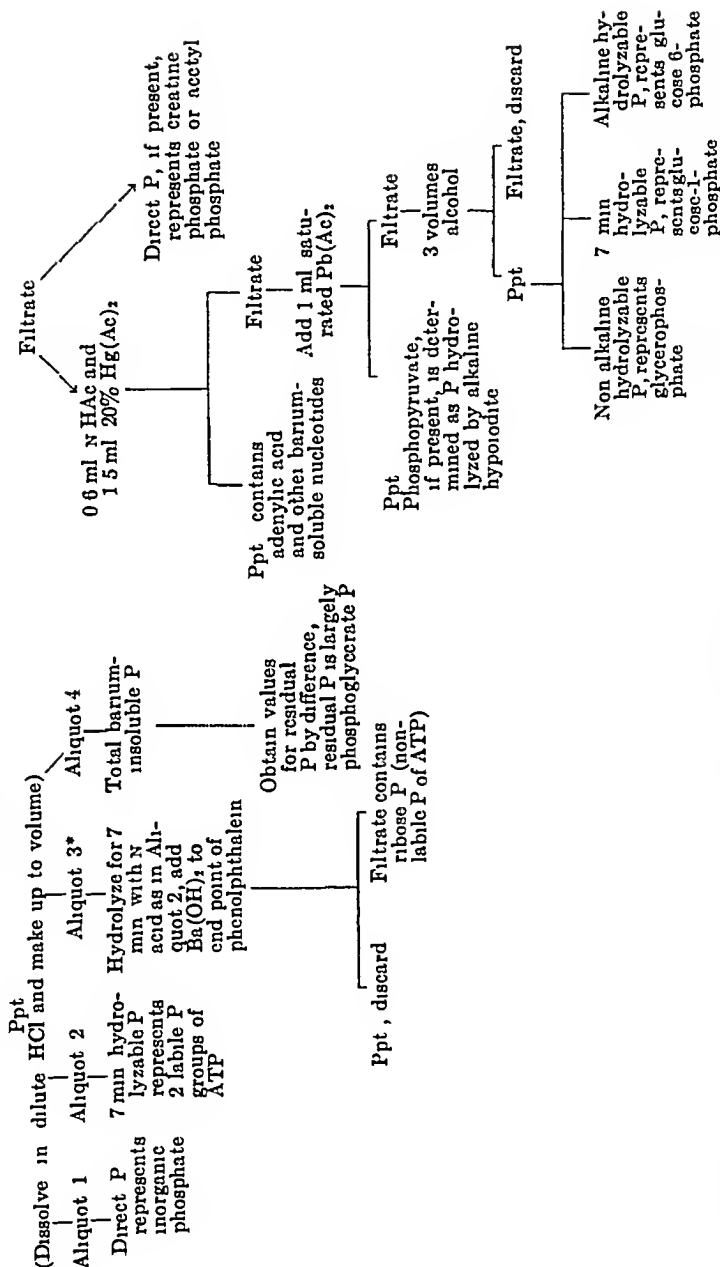
*Adenosine Triphosphate*<sup>3</sup>—The two labile phosphates of ATP are deter-

<sup>2</sup> Hereinafter designated as ATP

<sup>3</sup> There is apparently little adenosine diphosphate present in liver ordinarily Evidence for this is that the adenosine nucleotide fraction isolated as the mercury salts had an N P ratio of 5.3 However, it is possible that some adenosine diphos-



*Scheme for Separation of Organic Acid-Soluble Phosphates*  
 25 ml trichloroacetic acid extract  
 $\text{Ba}(\text{OH})_2$  to pH 8.2, faint pink to phenolphthalein



\* Necessary only in radioactive studies otherwise non labile P of ATP (adenosine triphosphate) can be determined by taking one half of the labile value

mined by hydrolyzing an aliquot of the fraction of insoluble barium salts with 1 N HCl for 7 minutes in a boiling water bath. The total amount of P in ATP is obtained by multiplying the labile P value by 3/2. ATP, when added to a trichloroacetic acid extract, is determined quantitatively by the use of the above procedure (see Table I).

A procedure for directly determining the non-labile phosphate of ATP was developed for use in connection with radioactive tracer studies. This is based on the solubility of the barium salt of ribose phosphate which is

TABLE I

*Recovery of Organophosphates Added to Trichloroacetic Acid Extracts of Rat Liver*

Compound	P added	P recovered
	mg	per cent
Adenosine triphosphate (labile P)	1 000	98.5
" " (non-labile P)	0 160	97.4
	0 500	96.0
	0 080	91.5
2,3-Diphosphoglyceric acid	0 917	97.0
	0 204	94.8
Muscle adenylic acid	0 786	95.4
	0 103	103.2
Phosphopyruvic "	0 682	96.4
	0 142	89.5
$\alpha$ -Glycerophosphate*	0 970	97.0
	0 115	94.1
Glucose-1-phosphate†	0 800	95.3
	0 135	90.5
Glucose-6-phosphate‡	0 580	91.0
	0 102	89.4

This table illustrates the recovery of various organic phosphate compounds when added separately to trichloroacetic acid extracts. The methods for separating these compounds are discussed in the text.

\* Determined as non-alkaline hydrolyzable P of alcohol precipitate

† Determined as 7 minute acid-labile P of alcohol precipitate

‡ Determined as alkaline hydrolyzable P of alcohol precipitate

formed during the acid hydrolysis of ATP. It has been established that adenylic acid is split into ribose phosphate and adenine during acid hydrolysis (11). The inorganic phosphate, which is present after 7 minutes acid hydrolysis of the fraction of insoluble barium salts, is precipitated with  $\text{Ba}(\text{OH})_2$  at pH 8.2. The filtrate should contain only ribose phosphate. A total P determination on the filtrate gives the non-labile P value 95 per

phosphate may be present under certain conditions. Hence values reported for ATP may at times represent mixtures of adenosine triphosphate and adenosine pyrophosphate.

cent recovery of the calculated value for the non-labile phosphate of ATP could be obtained by this method

We have confirmed Flock's findings (12) of the rapid autolysis of liver ATP

*Fructose Diphosphate*—One of the phosphate groups of fructose diphosphate is also easily hydrolyzable. Therefore, in order to make the determination of ATP valid, it is necessary to test the fraction of insoluble barium salts for fructose by the Roe method (13). This was applied and yielded no indication of the presence of fructose diphosphate in the liver in the varied physiological conditions which we have investigated.

*Residual Phosphate*—The residual P represents the phosphate in the fraction of insoluble barium salts which is not inorganic or ATP phosphorus. This value can be obtained by difference, namely, by subtracting the inorganic P and P of ATP from the total barium-insoluble P.

The residual P is very resistant to acid hydrolysis and is not hydrolyzed even after heating for 180 minutes. This property is characteristic of the phosphoglyceric acids. A positive test for glyceric acid was obtained on the fraction of insoluble barium salts by the use of the Rapoport naphthoresorcinol procedure (14). This positive test must be regarded with some reserve, since Neuberg and Lustig (15) have recently indicated that the naphthoresorcinol test is not specific for the phosphoglyceric acids. The small amount of this fraction isolated is significant in that it indicates that liver does not contain the large amounts of 2,3-diphosphoglycerate which are found in most mammalian red blood cells (16).

It is possible that this fraction contains some hexose monophosphates. The acid hydrolysis curve of the fraction of insoluble barium salts of rat liver was determined and found to be similar to that for dog liver which was obtained by Flock, Bollman, and Mann (4).

#### *Fraction of Soluble Barium Salts*

*Mercury Precipitate*—The Hg precipitate seems to consist mainly of nucleotide phosphate. No ATP appears in this fraction. Kerr (17) has prepared adenylic acid by precipitating it with Hg from an alkaline hydrolysate of ATP.

The acid hydrolysis curve of the Hg precipitate follows the typical curve for the hydrolysis of muscle adenylic acid (see Fig 1). The alkaline hydrolysis<sup>4</sup> of adenylic acid is compared with that of the Hg precipitate in Fig

<sup>4</sup> Alkaline hydrolysis of the organophosphate compounds was carried out essentially as described by Kurssanov (18). The hydrolysis was performed in 1 N NaOH in a boiling water bath. To avoid the interference of sodium with the color development in the determination of the resultant inorganic phosphate by the Fiske and Subbarow method, it is necessary to make the final acid concentration at least 0.4 N.

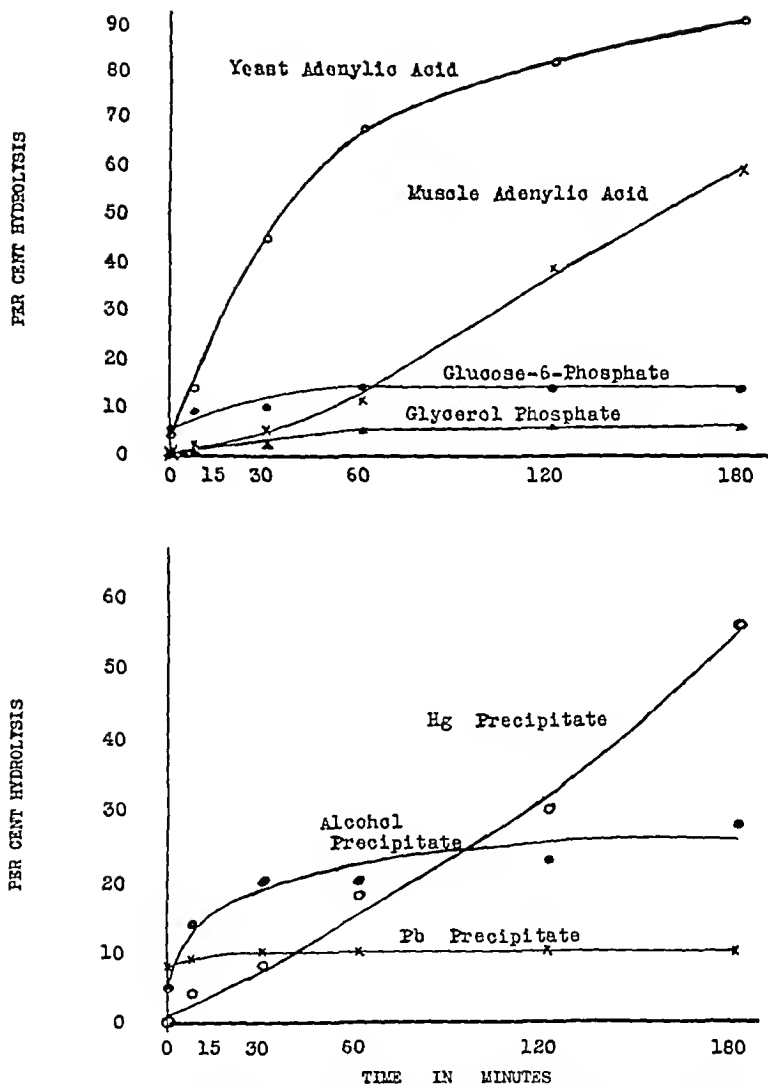


FIG 1 Acid hydrolysis curves of some known soluble barium salts of organophosphate compounds (upper) and of fractions of soluble barium salts of liver (lower)

2 The N P ratio of this fraction is close to 5 1 Adenylic acid when added to liver trichloroacetic acid extracts is recovered in the Hg precipi-

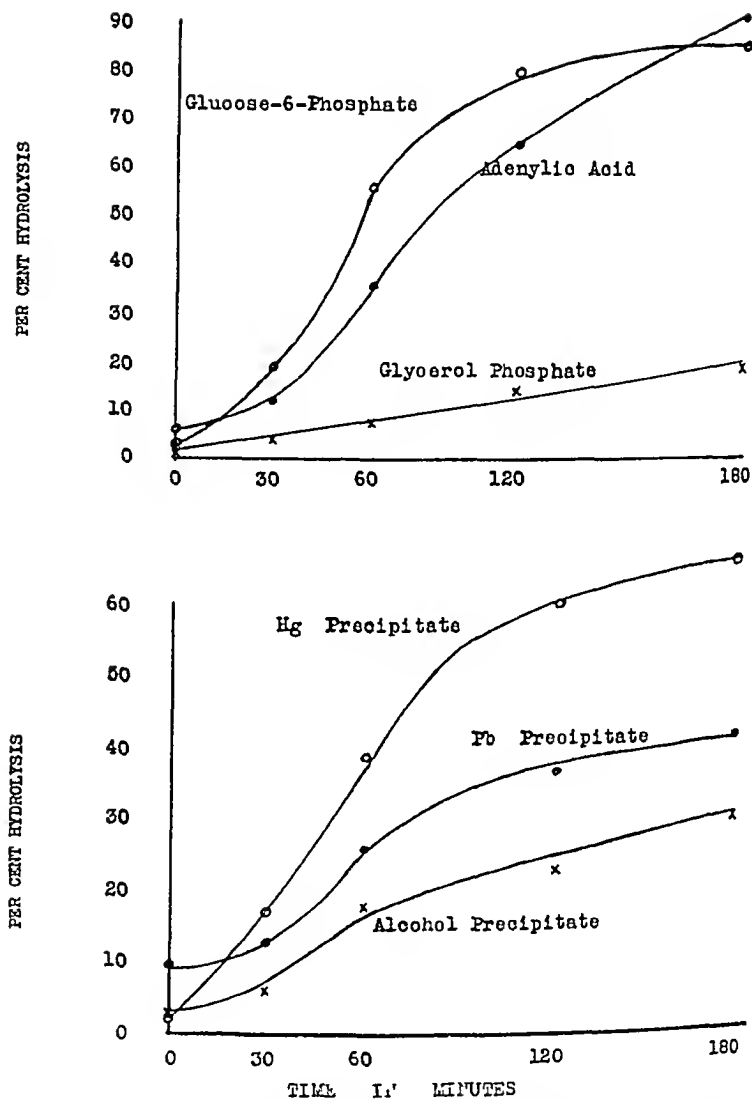


FIG 2 Alkaline hydrolysis curves of some known soluble barium salts of organophosphate compounds (upper) and of fractions of soluble barium salts of liver (lower)

tate A positive Bial's test was obtained on the fraction, indicating the presence of a pentose

*Lead Precipitate*—The Pb fraction contains but little of the acid-soluble phosphate. The directly determinable phosphate, which appears in this fraction, represents most of the inorganic phosphate which escapes precipitation by barium. Phosphopyruvic acid when added to trichloroacetic acid extracts can be recovered in the Pb precipitate. The phosphopyruvate can be distinguished from the other organophosphates in the Pb precipitate by the hypiodite method of Lohmann and Meyerhof (19). We have not been able to detect phosphopyruvate in normal rat liver.

It is also possible that a trace of glycerophosphate is present in this fraction. A negative test for pentoses was obtained, indicating that the Pb precipitate is devoid of nucleotide P. No reducing substances were found in this fraction.

*Alcohol Precipitate*—This fraction contains the bulk of the barium-soluble phosphates. Glycerophosphate constitutes a large part of this fraction. The acid and alkaline hydrolysis curves of the alcohol fraction are given in Figs 1 and 2. The small amount of easily hydrolyzable phosphate indicates the presence of some glucose-1-phosphate in liver. The low reducing values of the alcohol precipitate indicate that the glucose-6-phosphate levels of normal liver are quite low.

The alcohol precipitate gives positive acrolein and Denigés tests, which are indicative of the presence of glycerophosphate. The N/P ratio of the fraction is close to unity. A test for pentose was negative.

Kurssanov (18) has reported that glucose-6-phosphate is much more susceptible to alkaline hydrolysis than is  $\alpha$ -glycerophosphate. We have confirmed Kurssanov's findings (see Fig 2). Kurssanov, on the basis of the rates of alkaline hydrolysis of glucose-6-phosphate and  $\alpha$ -glycerophosphate, was able to determine the amounts of the two substances when they were present together in trichloroacetic acid extracts. We have used Kurssanov's method for the calculation of the amount of glucose-6-phosphate in the alcohol precipitate and have found that the content of the Robison ester P is approximately 5 mg per 100 gm of liver<sup>5</sup>. This value compares favorably with the values obtained by Cori, Cori, and Schmidt (21) for the hexose monophosphate content of rabbit liver.

*Directly Determinable P in Fraction of Soluble Barium Salts*—We have found very little directly determinable phosphate in the fraction of soluble barium salts from normal rat liver. This is in agreement with previous findings (4) in which creatine phosphate could not be detected as a constituent of liver.

\* Attempts to separate glucose-6-phosphate from glycerophosphate on the basis of the difference in solubility of their lead salts were unsuccessful. The lead salt of the Robison ester is completely soluble in pure solution. However, in the presence of glycerophosphate insoluble double salts of the hexose phosphate and glycerophosphate are formed. This has been reported previously by Smythe (20).

However, in rats injected with glucose, it was found that a considerable amount of directly determinable phosphate appeared in the fraction of soluble barium salts. That this direct phosphate was not inorganic phosphate was proved by the following experiment. Radioactive P in the form of  $\text{Na}_2\text{HPO}_4$  was added to a trichloroacetic acid extract of liver prepared from rats that had been injected with glucose. After fractionation with  $\text{Ba}(\text{OH})_2$ , it was found that the fraction of soluble barium salts contained only traces of radioactivity. However, it still contained relatively large amounts of directly determinable phosphate. This then indicated that the direct phosphate was due to an organic phosphate compound and not inorganic phosphate.

It was also found that no directly determinable phosphate remained after treatment of the trichloroacetic extract with magnesia mixture. This eliminated the possibility of the organic phosphate being creatine phosphate, since creatine phosphate is stable in magnesia mixture. The solubility and instability of the unknown compound are characteristic of Lipmann's acetyl phosphate (22). The quantity usually is about 3 per cent of the acid-soluble phosphate of the livers of rats administered glucose. It is possible that the compound is present in higher concentration, but is destroyed during the course of preparing the liver extracts. The appearance of an organophosphate with a possible energy-rich phosphate bond can be associated with the increased oxidation which occurs in liver following the administration of carbohydrate. Directly determinable phosphate was also found in the fraction of soluble barium salts after injection of insulin (see Table II).

*Nature of Phosphate Compounds Appearing When Liver Remains in Contact with Trichloroacetic Acid for Long Periods of Time*

Kay (7) observed an increase in the phosphate and nitrogen content of trichloroacetic acid extracts which had been in contact with liver for a period of 7 days. He attributed this increase to hydrolysis of phospholipid (lecithin). The increased phosphate ester was found to be barium-soluble and was assumed to be glycerophosphate, the increased nitrogen was thought to be due to an increase in choline content.

We considered it of value to reinvestigate the nature of the phosphate ester formed by allowing liver to remain in contact with trichloroacetic acid with the system of analysis given above. The results of the analysis of liver extract remaining in contact with trichloroacetic acid for 7 days are given in Table III.

The greater increase of nitrogen over phosphorus in the 7 day extract suggests that trichloroacetic acid causes the hydrolysis of other compounds besides lecithin. The data in Table III show that not only is there an

increase in the P of the alcohol precipitate (glycero-phosphate) but there also occurs a very marked elevation of the P in the mercury precipitate. A large percentage of the increased acid-soluble nitrogen also appears in the mercury precipitate. Less nitrogen is found in the alcohol precipitate. The N P ratio of the mercury precipitate of the zero time and 168 hour

TABLE II  
*Directly Determinable Phosphate in Fraction of Soluble Barium Salts*

Rats	Direct P	As per cent of total acid soluble P	Liver glycogen
	mg		per cent
Normal (fasted 12 hrs )	0.8	0.84	0.22
	0.4	0.41	0.42
	1.2	1.25	
	0.7	0.75	
Glucose-treated*	3.2	3.54	0.71
	2.7	2.66	0.80
	4.1	3.92	
	2.4	2.34	
Insulin-treated†	3.6	3.42	0.18
	2.1	1.90	0.27
	3.5	3.24	

\* Given 400 mg of glucose intraperitoneally, sacrificed 120 minutes after injection

† Given 4 units of insulin, sacrificed 90 minutes after injection

TABLE III

*Nitrogen and Phosphorus Partition in Filtrates from Trichloroacetic Acid Precipitates of Liver, in Contact with Trichloroacetic Acid for 7 Days*

Fraction	Filtrate from liver ppt in contact with trichloroacetic acid 0 hr			Filtrate from liver ppt in contact with trichloroacetic acid 7 days		
	N	P	N P	N	P	N P
	mg per 100 gm fresh tissue	mg per 100 gm fresh tissue		mg per 100 gm fresh tissue	mg per 100 gm fresh tissue	
Total acid-soluble	40.7	93.2	0.93	248.4	184.0	3.0
Hg ppt	23.2	11.7	4.4	152.8	69.0	4.7
Alcohol ppt	8.4	28.2	0.66	49.6	60.1	1.8

extracts do not differ greatly. An N P ratio of 5.1 is indicative of adenylic acid and it is most probable that some of the increased phosphate resulted from the hydrolysis of nucleic acids or nucleoproteins.

To test the above statement, experiments were carried out in which lecithin and nucleic acid were allowed to stand with trichloroacetic acid. The results are tabulated in Table IV.



It is apparent that the ester resulting from the trichloroacetic acid hydrolysis of lecithin is glycerophosphate (increase in alcohol and lead P). A large part of the nitrogen is found in the mercury precipitate, while only a small amount of the total acid-soluble P is present in the mercury precipitate. Little of the nitrogen is in the alcohol precipitate. It is quite possible, as suggested by Kay, that the nitrogen compound in the mercury precipitate is choline.

In contrast to lecithin, the hydrolysis of nucleic acid resulted in the appearance of large amounts of P and N in the mercury precipitate, the N/P ratio being 5.2/1. This would be in agreement with the findings on whole liver. Therefore, we feel justified in concluding that trichloroacetic acid not only causes an increased breakdown of insoluble phospholipid with

TABLE IV

*Nitrogen and Phosphorus Partition in Filtrates from Trichloroacetic Acid Hydrolysates of Lecithin and Nucleic Acid*

Fraction	Lecithin*			Nucleic acid (yeast)		
	N	P	N/P	N	P	N/P
	mg. per gm. lecithin	mg. per gm. lecithin		mg. per gm. nucleic acid	mg. per gm. nucleic acid	
Hg ppt	24.6	3.0	18.4	133.7	57.8	5.2
Pb "†	6.4	42.1	0.34	20.2	14.5	3.1
Alcohol ppt						

\* N/P ratio, 1.05/1

† In aqueous solutions, part of the glycerophosphate is precipitated as the lead salt. Therefore in order to obtain the total glycerophosphate content the P of the alcohol precipitate and lead precipitate has been added.

time, but also induces the hydrolysis of acid-insoluble nucleic acids and nucleoproteins.

#### DISCUSSION

It is of interest briefly to compare the methods that Rapoport, Leva, and Guest (6) have devised for the separation of the acid-soluble phosphates of liver with the scheme which we have developed.

The inorganic P values reported by Rapoport, Leva, and Guest (6) for normal fed rats are usually lower than our results. This is of interest, since our values are obtained from only the fraction of insoluble barium salts, whereas the Rapoport group determine inorganic phosphate directly on aliquots of trichloroacetic acid extracts. It would be expected that our values should be lower, since some direct P is found in the fraction of soluble barium salts. However, the discrepancies may be due to differences in the composition of the stock diets.

Rapoport, Leva, and Guest determine the labile phosphate (easily hydrolyzable) without previous separation. We have found that there are substances besides ATP in liver which possess easily hydrolyzable phosphate groups. This is indicated by the presence of labile P in our alcohol precipitate. Kosterlitz and Ritchie (23) recently have reported that small amounts of glucose-1-phosphate (Cori ester) are present in liver. Since the amount of easily hydrolyzable P which does not arise from ATP is quite small, it is quite likely that direct determination of labile P on liver extracts may give results which would not be misleading in interpreting the rôle played by ATP in liver reactions.

The mercury-insoluble P of Rapoport, Leva, and Guest differs from our mercury precipitate in that their fraction contains the non-labile P of ATP, whereas our fraction is devoid of any ATP phosphorus. Our mercury fraction apparently does not contain any non-nucleotide P, whereas the Rapoport precipitate does. We believe that our method is advantageous, particularly for radioactive studies, when it is necessary to measure the activity of the non-labile phosphate group of ATP. The Rapoport, Leva, and Guest method is advantageous in that it does not necessitate the removal of glycogen by mercuric sulfide.

While the method used by Leva and Rapoport (24) for the determination of glycerophosphate seems to be satisfactory for liver, we have recently found that the periodate reagent also causes the complete liberation of P from glucose-6-phosphate. Therefore it is possible that some of the P in the glycerophosphate fraction they report is really glucose-6-phosphate P. As yet we cannot judge with certainty where the alcohol-soluble P of the Cinnannat group falls in our scheme of analyses. We are also unable to tell where the P from our Pb precipitate will fall in the scheme of the Rapoport group.

We feel that the good points of our and Rapoport, Leva, and Guest's procedures can be incorporated into a scheme which can be used as a general system of analyses for the separation of acid-soluble phosphates.

We are indebted to Professors W Z Hassid and H A Barker of the Division of Plant Nutrition and to Professor F W Allen and Dr A C Walker of this laboratory for the supply of some of the compounds used in these experiments and for their criticism of various aspects of this work.

#### SUMMARY

1 A scheme is given for the separation of the acid-soluble phosphates of liver.

2 Experiments have been carried out to determine the nature of the phosphate esters in certain of the liver fractions.

3 The acid and alkaline hydrolysis curves of various known organophosphates have been determined and compared with certain isolated liver fractions

4 It has been found that adenylic acid or some closely related nucleotide, as well as glycerophosphate, is liberated when trichloroacetic acid precipitates of liver tissue remain in contact with the acid for long periods of time

5 The scheme of analysis which we have developed is compared with that developed by Rapoport, Leva, and Guest for the separation of acid-soluble phosphates of liver

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# STUDIES WITH RADIOACTIVE PHOSPHORUS OF THE CHANGES IN THE ACID-SOLUBLE PHOSPHATES IN THE LIVER COINCIDENT TO ALTERATIONS IN CARBOHYDRATE METABOLISM

## II THE EFFECT OF GLUCOSE, INSULIN, AND OF CERTAIN METABOLIC INHIBITORS

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(Received for publication, August 4, 1944)

Researches of recent years have demonstrated the importance of phosphate intermediates in the carbohydrate metabolism of the liver. Cori, Cori, and Schmidt (1) have reported the presence of phosphorylase in liver preparations. The glucose-1-phosphate (Cori ester) produced by the action of phosphorylase is reversibly converted to glucose-6-phosphate by the enzyme phosphoglucomutase (2). It has been suggested that liver phosphorylase and phosphatase play predominant rôles in the conversion of liver glycogen to blood glucose (3). Liver extracts are capable of phosphorylating glucose through the agency of the adenylic acid system. Cori (4) has stressed the importance of aerobic phosphorylations in the synthesis of glycogen from glucose.

The present investigation was undertaken to determine the relationship between the metabolic processes occurring in the intact liver with the phosphorylation reactions that have been established with tissue slices and enzyme extracts.

By employing phosphate labeled with the radioactive isotope  $P^{32}$  as a tracer it was possible to discern the turnover of phosphate fractions of the liver in which there was little or no change in the total concentrations.

### *Methods*

The organic acid-soluble phosphate fractions were separated according to the procedures described in Paper I of this series (5). The phosphorus content of each fraction was determined colorimetrically by the Fiske and Subbarow method, as already described.

The radioactivity of the phosphate fractions was determined in the manner described below. Inorganic phosphate was precipitated from an aliquot

\* The material of this paper was taken from a thesis submitted by N. O. Kaplan to the Graduate Division of the University of California in partial fulfillment of the requirements for the degree of Doctor of Philosophy, October, 1943.

of the dissolved precipitate of the insoluble barium salts as magnesium ammonium phosphate<sup>1</sup> with the magnesia mixture of Sacks and Sacks mentioned by Cori and Cori (7). It is usually necessary to add carrier phosphate to attain complete precipitation. The precipitate was centrifuged down and the supernatant discarded. After being washed, the precipitate was dissolved in dilute HCl and transferred to tin ointment capsules. After evaporation, the radioactivity was measured on the Lauritsen electroscope or on a Geiger-Müller counter with the mica window tube described by Copp and Greenberg (8).

The labile  $P^{32}$  in the  $ATP^{32}$  was determined by magnesium precipitation after an aliquot of the fraction of insoluble barium salts was hydrolyzed for 7 minutes in a boiling water bath. Determination of the non-labile phosphate was carried out by reprecipitating a 7 minute-hydrolyzed aliquot with  $Ba(OH)_2$ . The non-labile phosphate which is converted to ribose phosphate remains in the filtrate. The value for the residual fraction of barium salts was determined by difference. Suitable aliquots of the Hg, Pb, and alcohol precipitates were used for  $P^{31}$  and  $P^{32}$  determinations.

The experiments were carried out on rats of the Long-Evans strain weighing between 175 and 275 gm. Radioactive phosphorus was injected intraperitoneally in the form of  $Na_2HPO_4$  in amounts of between 0.5 and 1.0 mg.

#### EXPERIMENTAL

##### *Distribution of $P^{32}$ in Acid-Soluble Phosphate Fractions of Livers from Rats in Postabsorptive State<sup>3</sup>*

These experiments were carried out on rats fasted for 12 hours. The animals were sacrificed at varying intervals after injection of the  $P^{32}$ . A

<sup>1</sup> In order to obtain complete precipitation of the inorganic phosphate, it is necessary to place the reaction mixture in a refrigerator for a minimum period of 6 hours. It is necessary to maintain the mixture at about pH 8, as ATP is slowly hydrolyzed in alkaline solution at 0° (6). In the beginning of this work the strychnine method, which has certain advantages, was used for precipitating inorganic phosphate. The method was abandoned when it was observed that partial hydrolysis of the labile phosphates of ATP ensued.

<sup>2</sup> To save space, ATP is used as an abbreviation for adenosine triphosphate. In some instances the fraction may represent a mixture of adenosine triphosphate and adenosine diphosphate (see (5) foot-note 3).

<sup>3</sup> The phosphorus fractions of the blood contained in the livers are included in all our data. Perfusion of the liver to eliminate the blood is not practicable in connection with studies on the turnover of the liver phosphates. The lability of the liver organic phosphates, particularly of ATP, during autolysis demonstrates that perfusion would have induced changes in these compounds that could greatly obscure the observed findings. It is the opinion of the writers that the organic phosphates from the blood in the liver have little if any influence on the significance of the studies on the

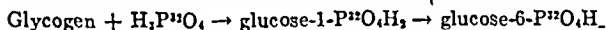
summary of the results at two different time intervals is given in Tables I and II

The values of the relative and specific activities<sup>4</sup> of the different fractions were calculated. Curves of the change of the relative activities with time of this group of animals are given in Fig. 1. The specific activity represents the  $P^{32}$  :  $P^{31}$  ratio of each fraction, whereas the relative activity is the ratio of the  $P^{32}$  of the fraction to the total acid-soluble  $P^{31}$ . The specific and relative activities of the total acid-soluble phosphate are of course identical.

Several of the phosphate fractions are undoubtedly heterogeneous, and under these conditions values of specific activity are of little importance because of the different rates of turnover of the individual components of a fraction. However, the relative activity is not necessarily dependent on purity, and changes which cannot be noticed in the specific activity can be detected in the values of the relative activity. The relative activity is a measure of the distribution of  $P^{32}$  in the different acid-soluble phosphate fractions and it is useful in establishing the time relationship of the different phosphate fractions.

In the postabsorptive state, the total acid-soluble  $P^{32}$  attains its maximum concentration in the liver at 105 to 110 minutes after its injection. The inorganic  $P^{32}$  entering the liver is rapidly esterified. The greatest uptake of  $P^{32}$  is by the alcohol and the residual fractions of the insoluble barium salts.

Since the alcohol precipitate consists partially of glucose-6-phosphate, the  $P^{32}$  entering the liver may have undergone the reactions shown below



Evidence on this point was derived from the alkaline hydrolysis of the alcohol-insoluble fraction (see (5)). The results are given in Table III. The alkali-labile P (probably hexose monophosphate) was found to have a much higher specific activity than the alkali-non-labile P (mainly glycerophosphate). The low specific activity of the alkali-non-labile fraction indicates that the rate of turnover of glycerophosphate is much lower than that of the hexose monophosphate.

The large amount of radioactivity found in the residual fraction of the

acid-soluble phosphates of this organ. From measurement of the hemoglobin content, it appears that liver contains 3 to 4 ml. of red corpuscles per 100 gm. of fresh tissue (9). This amount of blood could only contribute, for example, about 1 per cent to the labile P of the ATP of the liver. A differentiation cannot at present be made into intra- and extracellular phosphate. Fortunately, this is of importance only for the inorganic phosphate. This differentiation is not essential for the present investigations, since no attempt was made to determine precursors or turnover rates from the data for specific activity.

<sup>4</sup> For a discussion of the significance of specific activities see Zilversmit, Entenman, and Fishler (10).



insoluble barium salts is difficult to explain. This fraction seems to consist largely of phosphoglyceric acids. It is significant that the labile  $P^{32}$  in ATP attained its maximum after the maximum concentration of  $P^{32}$  in the other

TABLE II

*Distribution of  $P^{31}$  and  $P^{32}$  in Acid Soluble Phosphates of Liver 200 Minutes after Administration of  $NaH^{32}PO_4$*

$P^{31}$  values are in mg of P per 100 gm of fresh liver,  $P^{32}$  values are in parts per 1000 of the administered dose per 100 gm of fresh liver. The measure of variability is the standard error of the mean.

	No of rats	Total acid soluble		Inorganic		Labile P of adenosine triphosphate		Hg ppt		Alcohol ppt	
		$P^{31}$	$P^{32}$	$P^{31}$	$P^{32}$	$P^{31}$	$P^{32}$	$P^{31}$	$P^{32}$	$P^{31}$	$P^{32}$
Controls fasted 12 hrs	4	95.1 $\pm 1.9$	102.3 $\pm 2.1$	23.0 $\pm 1.4$	41.7 $\pm 3.1$	10.8 $\pm 1.1$	17.8 $\pm 1.2$	9.7 $\pm 1.1$	11.9 $\pm 1.2$	26.0 $\pm 0.88$	20.3 $\pm 1.5$
Glucose (400 mg intraperitoneally)	3	103.8 $\pm 1.4$	128.0 $\pm 4.8$	20.8 $\pm 0.37$	33.6 $\pm 1.8$	19.7 $\pm 0.72$	14.8 $\pm 1.3$	13.4 $\pm 0.36$	13.0 $\pm 0.67$	33.3 $\pm 0.82$	29.7 $\pm 1.5$
Insulin (4 units intraperitoneally)	3	110.3 $\pm 1.9$	200.4 $\pm 8.8$	29.5 $\pm 0.98$	57.1 $\pm 2.6$	16.1 $\pm 0.61$	18.3 $\pm 2.7$	14.3 $\pm 0.41$	23.4 $\pm 1.5$	31.3 $\pm 0.65$	24.8 $\pm 1.9$
Glucose + insulin (as above)	3	117.4 $\pm 1.8$	200.1 $\pm 10.2$	26.9 $\pm 0.99$	50.0 $\pm 3.3$	22.2 $\pm 0.69$	28.0 $\pm 1.4$	13.3 $\pm 0.44$	19.7 $\pm 1.1$	34.4 $\pm 0.92$	32.7 $\pm 2.5$

CONTROL - RELATIVE ACTIVITY

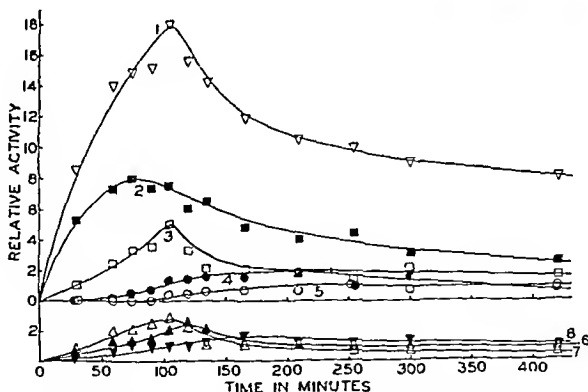


FIG 1 Relative activity curves of liver phosphorus fractions of control rats. Curve 1, total acid-soluble P, Curve 2, inorganic P, Curve 3, alcohol precipitate, Curve 4, labile P in adenosine triphosphate, Curve 5, non-labile P in adenosine triphosphate, Curve 6, Hg precipitate, Curve 7, residual P, Curve 8, Pb precipitate. The points at 105 minutes are an average of ten determinations.



fractions had been reached. This suggests the possibility of transfer of  $P^{32}$  from the various esters to adenylic acid. Since only an energy-rich phosphate group can be transferred to the adenylic acid system, the immediate donor cannot be glucose-6-phosphate, phosphoglyceric acid, or glycerophosphate (11). The donors may be high energy phosphate compounds resulting from the further oxidation of these esters.

The low radioactivity of the non-labile phosphate in ATP (Fig 1) is evidence in support of the concept that only the labile phosphate of ATP takes part in phosphate transfer reactions. The esters in the Pb and Hg precipitates apparently also undergo renewal at a fairly rapid rate.

TABLE III

*Partition of  $P^{32}$  after Alkaline Hydrolysis of Barium-Soluble, Alcohol-Insoluble Phosphorus Fraction of Rat Liver*

The values are the means of analyses on four rats each.  $P^{31}$  values are in mg of P per 100 gm of liver,  $P^{32}$  values are in parts per 1000 of the administered dose per 100 gm of liver.

	Time after $P^{32}$ adminis- tration	$P^{31}$			$P^{32}$			Specific activity = $P^{32}/P^{31} \times 10$		
		Total alco- hol	Alkali labile	Alkali non labile	Total alcohol	Alkali labile	Alkali non labile	Total alcohol	Alkali labile	Alkali non labile*
	min									
Postabsorptive state	110	28.8	8.7	20.1	48.0	20.0	28.0	16.7	22.9	13.9
After 400 mg glucose	110	30.6	9.1	21.5	24.4	6.5	17.9	8.0	7.2	8.3
" "	210	34.9	9.7	25.2	32.8	8.4	24.4	9.4	8.7	9.7

\* The alkali-non-labile phosphate is the fraction which is not hydrolyzed by 5 per cent NaOH in 180 minutes.

### *Effect of Glucose Injection*

In this series of experiments, rats fasted for 12 hours were injected with 400 mg of glucose in isotonic solution and then 20 minutes afterwards they were injected with  $Na_2HP^{32}O_4$ . The results are given in Tables I to III and the curves of the relative activities are plotted in Fig 2.

There is a significant increase in both the total acid-soluble  $P^{31}$  and  $P^{32}$  following the administration of glucose. Therefore the values for specific activity of the total acid-soluble P are only slightly higher than in the controls.

The rise in the total acid-soluble phosphate is due, at least in part, to an increase in the  $P^{31}$  content of the alcohol precipitate. Alkali hydrolysis showed that the increased phosphate was mainly in the alkali-non-labile

fraction and therefore presumably glycerophosphate. In contrast to the controls (Table III), the specific activities of the alkali-labile and alkali-non-labile P fractions are quite similar in the glucose-treated animals.

It is apparent from examination of the curve that injection of glucose causes a marked change in the time pattern of the phosphate metabolism of the liver. In contrast to the control animals a great deal of  $P^{32}$  is rapidly incorporated into the labile groups of ATP. Inorganic  $P^{32}$  disappears more rapidly in the glucose-treated animals than in the controls. Inorganic  $P^{31}$  also decreased as a result of introduction of glucose.

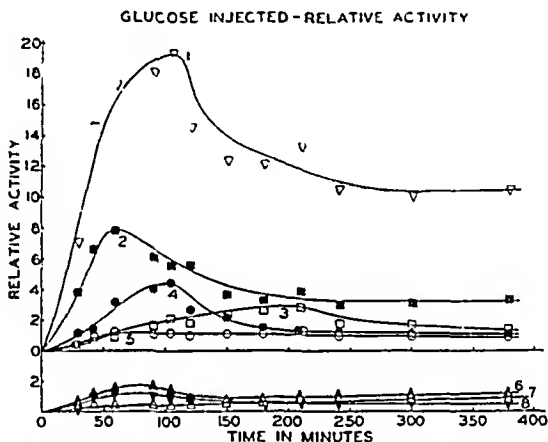


Fig 2 Relative activity curves of liver phosphorus fractions of glucose-injected rats. Curve 1, total acid-soluble P, Curve 2, inorganic P, Curve 3, alcohol precipitate, Curve 4, labile P in adenosine triphosphate, Curve 5, non-labile P in adenosine triphosphate, Curve 6, Hg precipitate, Curve 7, residual P, Curve 8, Pb precipitate. The points at 105 minutes are an average of eight determinations.

Examination of Fig 2 shows that the maximum relative activity in the alcohol precipitate is attained after the labile  $P^{32}$  of ATP has reached its maximum value. This would indicate a possible transfer of P from ATP to glucose with formation of glucose-6-phosphate, a reaction that has been shown to take place *in vitro*. It is noteworthy that there is little increase in the quantity of glucose-6-phosphate (alkali-labile fraction) after glucose administration. The increase in the radioactivity of the glycerophosphate (alkali-non-labile fraction) probably also arises from transfer of P from ATP.

It is of interest that there is also considerable radioactivity in the non-labile P of ATP. It has generally been accepted that the non-labile P

It is interesting to note that the values of the residual fraction of insoluble barium salts lie on a curve intermediate between the curves of the normal and glucose-treated animals. The slow increase in relative activity in the first part of the curve resembles the curve of the glucose-treated animals, whereas the latter part is similar to the curve of the controls. As in the case of the alcohol-insoluble fraction, the relative activity of the residual fraction of insoluble barium salts does not approach the maximum value reached in the control group.

Insulin produced little change in the Pb fraction.

### *Effect of Glucose Plus Insulin Administration*

In this series, the animals were injected with the isotonic glucose solution and 4 units of insulin 20 minutes after receiving the radioactive phosphate. The results are given in Tables I and II.

The combination of glucose and insulin produces the greatest observed increases in the total acid-soluble  $P^{32}$  and the  $P^{32}$  of the labile phosphate of ATP. The increase in inorganic  $P^{32}$  is less than that produced by insulin alone. The increase in  $P^{32}$  in the labile groups of ATP in the insulin glucose-administered animals may represent an enhanced oxidation of the administered glucose. Adding significance to the above is the observation of Sacks (14), that in resting muscle administration of glucose and insulin results in an acceleration of the turnover rates of ATP and creatine phosphate. These findings constitute evidence that insulin influences the production of energy-rich phosphate bonds.

Administration of insulin, of glucose, and of both together produces elevations in both the  $P^{31}$  and  $P^{32}$  contents of the alcohol and mercury precipitates. The maximum increases take place 3 hours after administration.

### *Effect of Sodium Fluoride*

The existence of potent phosphatase activity in the liver satisfactorily explains the small amount of glucose-6-phosphate found in the trichloroacetic acid extracts of this organ. Ostern and Holmes (3) reported that autolysis of liver glycogen in the presence of NaF resulted in the accumulation of the Robison ester. When glucose-1-phosphate was added to liver extracts in the presence of NaF, glucose-6-phosphate could be recovered as one of the reaction products (15).

The effect of NaF on the liver of the intact animal is described in the experiments reported below.

Sodium fluoride was injected intraperitoneally. No noticeable changes in the liver phosphates occurred until the amount of fluoride injected ap-

proached the lethal dose (75 mg of NaF per 100 gm of rat<sup>5</sup>) The results of fluoride injection on the partition of the liver phosphates are summarized in Table IV

The increase in the P and the reducing values of the alcohol-insoluble fraction induced by the fluoride indicate that it caused the accumulation of an ester which appears to be glucose-6-phosphate, perhaps by inhibiting the activity of liver phosphates There is an increase in the percentage of alkali-labile phosphate in the alcohol precipitate, which is further evidence that the ester accumulating is glucose-6-phosphate The rise in

TABLE IV

*Effect of Administration of Sodium Fluoride on Acid-Soluble Phosphates of Liver*

All animals were fasted for 12 to 16 hours before injection The numbers in parentheses refer to the number of determinations The measure of variability is the standard error of the mean

	Blood sugar	Total acid soluble P	Inorganic P	Alcohol ppt P	Reducing sugar in alcohol ppt	Maximum glucose 6- phosphate calculated from reduc- ing values*
	mg per 100 ml	mg per 100 gm fresh liver	mg per 100 gm fresh liver	mg per 100 gm fresh liver	mg per 100 gm fresh liver	mg per 100 gm fresh liver
Control, given 2 ml saline intraperitoneally	99.5 ±4.4 (4)	93.5 ±1.4 (6)	24.2 ±0.62 (6)	26.5 ±0.69 (6)	24.5 ±7.1 (5)	6.1 ±1.3 (5)
Fluoride-treated, given 75 mg NaF per 100 gm body weight in 2 ml solution 25- 30 min prior to removal of liver	67.0 ±7.9 (6)	100.1 ±0.95 (7)	22.1 ±0.70 (8)	34.0 ±1.31 (8)	67.9 ±6.9 (7)	16.1 ±1.3 (7)

\* These values are calculated on the basis that hexose-6-phosphate gives only 60 per cent of the theoretical reducing value when determined by the Miller-Van Slyke method

glucose-6-phosphate is associated with a decrease in blood sugar level The fluoride apparently did not produce any increase in the amount of glucose-1-phosphate in the liver

The results can be accepted as evidence that in the intact liver glycogen is converted to blood sugar through the following steps: glycogen → glucose-1-phosphate → glucose-6-phosphate → glucose (blood)

Experiments also were carried out on the influence of fluoride on the

<sup>5</sup> The animals lived for about 25 to 35 minutes when given the above dose The reaction to fluoride is quite interesting The rats tend to have convulsions, following which there occurs a general paralysis and a labored deep respiration

partition of labeled phosphate in the liver Inasmuch as rats live only 25 to 35 minutes after injection of NaF, it was introduced at varying intervals after the administration of the radioactive phosphate in order to observe the influence of time The rest of the procedure was the same as in the experiments described above Significant changes occurred only in the total acid-soluble, inorganic, and alcohol-insoluble phosphate fractions The changes induced in these fractions are recorded in Table V

TABLE V  
*Effect of Administered Sodium Fluoride on Distribution of Radioactive Phosphate in Liver*

	Time after administering $P^{32}$ min	Total acid soluble			Inorganic				Alcohol ppt			
		$P^{31}$ *	$P^{32}$ †	$Sa$ ‡	$P^{31}$	$P^{32}$	$Sa$	$Ra$ §	$P^{31}$	$P^{32}$	$Sa$	$Ra$
Controls	60	95.5	141.2	14.9	21.6	68.6	31.7	7.3	28.3	27.0	9.5	2.8
	75	101.7	151.0	15.1	26.5	85.6	32.3	8.4	27.6	36.6	13.3	3.6
	95	98.0	156.8	15.9	24.1	75.2	31.2	7.7	27.7	39.8	14.8	4.1
	115	94.5	158.0	16.8	25.9	60.8	23.4	6.4	28.5	33.7	11.8	3.6
	135	93.0	125.9	13.5	22.9	54.8	23.1	5.8	27.8	20.4	7.4	2.2
	185	101.0	94.6	9.4	21.7	34.8	16.0	3.5	26.6	18.8	7.1	1.9
NaF administered	60	104.8	178.2	17.0	24.0	42.5	17.7	4.1	36.4	53.5	14.7	5.1
	75	105.1	159.0	15.1	24.3	49.5	21.3	4.7	32.7	45.0	13.7	4.3
	95	101.0	193.2	19.3	24.2	51.6	21.4	5.1	35.0	68.5	20.8	7.2
	115	106.5	213.8	20.0	23.8	40.9	17.8	3.9	33.4	76.8	22.9	7.4
	135	103.8	163.2	15.7	26.0	53.2	20.5	5.2	31.2	27.3	8.7	2.7
	185	101.2	109.2	10.8	24.8	29.0	11.6	3.1	36.0	22.8	6.4	2.3

\*  $P^{31}$  values are in mg of P per 100 gm of fresh liver

†  $P^{32}$  values are in parts per 1000 of the administered dose per 100 gm of fresh liver

‡  $Sa$  = specific activity =  $(P^{32}/P^{31}) \times 10$  of fraction

§  $Ra$  = relative activity =  $(P^{32} \text{ of each fraction}) / (\text{total acid soluble } P^{31}) \times 10$

|| Dose used = 75 mg per 100 gm of rat Rats were given the fluoride 25 to 30 minutes prior to removal of the liver

The results show that there is a marked increase in the acid-soluble  $P^{32}$  in contrast to only a slight increase in the  $P^{31}$ . This suggests the possibility that some  $P^{32}$ , which normally leaves the liver, is retained in the form of glucose-6-phosphate, because some of the additional  $P^{32}$  can be accounted for by the increase in the alcohol-insoluble fraction. There is a decrease in the specific and relative activities of the inorganic phosphate. This is an indication that the fluoride prevents the liberation of inorganic  $P^{32}$  from some organic precursor.

$P^{32}$  changes are noticeable only in those animals injected with NaF before

the total acid-soluble  $P^{32}$  in the liver has reached its maximum concentration. This observation suggests that only the labeled phosphate which has entered the liver is affected by the fluoride. It offers further evidence for the uptake by the liver of inorganic phosphate from the blood plasma and its conversion into organic form therein.

It is interesting that sublethal doses of fluoride produce a hyperglycemia instead of the hypoglycemia observed with lethal doses. This paradox may be due to a high degree of inhibition of the reactions which prevent the uptake of sugar by muscle and a low degree of inhibition of the liver phosphatase by the lower dosage. This sequence of events would lead to an increased blood sugar. Fluoride, in lethal doses, presumably inhibits the liver phosphatase and hence prevents the liver from contributing glucose to the blood stream.

In contrast to Ostern, Heibert, and Holmes, who observed an inhibition of phosphatase activity in liver brei (15), Cori, Cori, and Schmidt (1) were unable to demonstrate any inhibitory effect of fluoride in their liver preparation. These differences may be due to the fact that the Ostern group used fasted rabbit liver, whereas the Cori group used the livers of well fed rabbits. We have observed that administration of NaF only had an effect on the liver phosphates of fasting rats.

#### *Effect of Various Inhibitors on Formation of Adenosine Triphosphate*

From Fig. 2 it is evident that the ATP values are indicative of the changes induced by glucose administration. Consequently it was felt that a study of the factors influencing the level of ATP would be of importance in elucidating the nature of the mechanisms in the liver concerned with the utilization of glucose.

The results obtained in the partition of the liver phosphates of the rat after injection of glucose and various metabolic inhibitors are summarized in Table VI.

**Iodoacetate**—Sodium iodoacetate was injected 60 minutes before the animal was sacrificed. It was prepared just prior to injection by neutralizing iodoacetic acid with NaOH. There were no fatalities with the concentration used (10 mg per 100 gm of body weight).

Iodoacetate apparently does not interfere with the phosphorylation processes of the liver, although the dosage employed is known to produce metabolic disturbances (16). This was evidenced by the animals showing the characteristic symptoms of iodoacetate poisoning. It is known that iodoacetate is a powerful inhibitor of anaerobic glycolysis, but has little effect on metabolism under aerobic conditions. The fact that the liver is a highly aerobic organ may be the explanation for the lack of inhibition by the iodoacetate.

*Malonate*—Sodium malonate, prepared by neutralizing malonic acid to pH 6.8 with NaOH, was introduced 60 minutes prior to the removal of the liver.

The malonate produced some decrease in the amount of labile  $P^{32}$  of ATP and an accumulation of inorganic  $P^{31}$  and  $P^{32}$ .

Stare and Baumann (17) have reported that malonate abolishes the hypoglycemic effect of insulin, they also showed that malonate alone causes a temporary hyperglycemia. Our findings suggest that the tricarboxylic acid cycle (18) is involved in the phosphorylation processes of the intact liver.

TABLE VI

*Effect of Various Inhibitors on Acid-Soluble Phosphate Distribution of Rats Receiving Glucose*

All rats were fasted 12 hours before treatment. They were sacrificed 110 minutes after receiving the  $P^{32}$  in the form of  $\text{Na}_2\text{HPO}_4$ .  $P^{31}$  values are in mg of P per 100 gm of fresh liver,  $P^{32}$  values are in parts per 1000 of the administered dose per 100 gm of liver. The measure of variability is the standard error of the mean.

Treatment in addition to 400 gm glucose	No of rats	Total acid soluble		Inorganic		Labile P of adenosine triphosphate		Alcohol ppt	
		$P^{31}$	$P^{32}$	$P^{31}$	$P^{32}$	$P^{31}$	$P^{32}$	$P^{31}$	$P^{32}$
None	6	102.2 ±1.1	198.8 ±6.2	20.8 ±0.37	54.8 ±2.4	21.2 ±0.39	46.5 ±2.4	27.8 ±0.89	25.7 ±1.3
10 mg iodoacetate per 100 gm rat weight	5	103.8 ±1.7	191.1 ±4.8	20.7 ±0.67	66.6 ±2.5	20.2 ±1.4	46.6 ±2.8	27.8 ±0.84	26.9 ±2.9
75 mg NaF per 100 gm rat weight	4	98.5 ±0.84	186.4 ±5.2	24.7 ±0.94	83.2 ±4.6	13.2 ±0.50	24.5 ±0.81	28.5 ±1.0	23.4 ±1.1
1 ml 1 M malonate per rat	5	96.0 ±1.9	175.1 ±6.6	24.6 ±0.55	98.7 ±2.6	14.6 ±0.59	27.6 ±1.3	26.9 ±0.65	21.5 ±0.55
75 mg phlorrhizin per 100 gm rat weight	4	103.0 ±1.7	196.4 ±10.0	25.5 ±1.3	133.7 ±5.9	12.4 ±0.58	18.6 ±1.3	26.7 ±0.54	16.6 ±0.87

*Sodium Fluoride*—In this series the rats were first injected with  $\text{Na}_2\text{H}^{32}\text{O}_4$ , 20 minutes later they were injected with glucose, 50 minutes after glucose administration they were injected with NaF and then sacrificed 30 minutes later.

There is an appreciable diminution of the labile  $P^{31}$  and  $P^{32}$  of ATP in the fluoride-treated rats. However, the specific activities remain about the same as that of the controls. There is no increase in the  $P^{32}$  of the alcohol precipitate of the rats treated with glucose and fluoride, as is the case in the livers of fasting animals injected with NaF (see Table V). Considerable radioactivity was found in the residual fraction of the barium salts of the rats given glucose and fluoride. These differences further indicate that injection of glucose causes a shift in the reactions of the liver.

*Phlorhizin*—For these experiments, rats were injected with phlorhizin dissolved in propylene glycol, as described by Weissberger (19), 60 minutes prior to the removal of the liver

Weissberger found that phlorhizin produced little alteration in the total  $P^{32}$  of the liver. Rapoport and coworkers (20) found a lowering of the specific activities of the labile P of ATP of the kidney after phlorhizin administration. Our results indicate little change in the total acid-soluble  $P^{32}$ , but the changes in the ATP values are striking. The phlorhizinized rats were the only poisoned animals that showed a lowering of both the specific and relative activities of the labile phosphate groups of ATP. There was also an increase in the inorganic  $P^{32}$ . This suggests a decrease of phosphorylation processes, which is understandable, because phlorhizin is known to be a potent inhibitor of phosphorylation. However, loss of glucose through the kidneys may be a contributing factor in the lowered phosphate fixation in the liver, since blood sugar changes are primarily responsible for an alteration in liver metabolism.

#### DISCUSSION

In the main, the experimental results reported here offer *in vivo* support to the phosphate cycle in carbohydrate metabolism that has been derived from *in vitro* studies.

The phosphorolysis of glycogen probably accounts for the major part of the  $P^{32}$  incorporated into the organic acid-soluble components of the livers of the rats on normal regimens. In this reaction inorganic phosphate is taken up enzymatically. The phosphorolysis of liver glycogen is apparently a continuous process in the intact animal. This is illustrated by the increase in glucose-6-phosphate after fluoride injection.

The phosphorolysis of glycogen is the only reaction in the carbohydrate-phosphate cycle in which orthophosphate is esterified that is definitely known to be enzymatic. All other reactions leading to the uptake of inorganic phosphate are associated with oxidations (4). Of outstanding importance is the intervention of phosphate in the oxidation of phosphoglyceraldehyde in conjunction with coenzyme I. The phosphate taken up during oxidation is transferred by the adenylic acid system to suitable phosphate acceptors (21, 22). Iodoacetate is known to inhibit this reaction. In the present experiments, however, no inhibition of ATP formation was caused by iodoacetate. Barker, Shorr, and Malam (23) have reported the aerobic oxidation of glucose in iodoacetate-treated muscles. Fazekas and Himwich (24) found that iodoacetate is much more toxic at low than at normal oxygen pressures. It appears probable that inorganic phosphate is fixed in the liver by different pathways besides the Embden-Meyerhof-Parnas scheme.

Recent evidence has indicated that certain steps in the tricarboxylic



acid cycle are coupled with the uptake of phosphate. The phosphorylated intermediates have as yet not been established, although a number of possible intermediates have been suggested. Ochoa (25) and Cori and his colleagues (12, 26) have shown that the energy derived from oxidation in the tricarboxylic acid cycle is used for phosphorylations. The inhibition of ATP formation by malonate observed by us may then be attributed to inhibition of the formation of high energy phosphates from the tricarboxylic acid cycle.

The evidence from our data and the work on kidney by Rapoport and coworkers (20) clearly indicates that phlorhizin inhibits phosphorylation *in vivo*. Cori, Colowick, and Cori (27) have reported inhibition of phosphorylase activity by phlorhizin. Kalckar (28) has shown that phlorhizin abolishes phosphorylation in kidney extracts, and, in particular, that phlorhizin prevents the oxidation of glyceraldehyde phosphate. It appears that phlorhizin inhibition is manifested through repression of phosphate uptake in one or several reactions. It has been suggested by Beck (29) that phlorhizin acts by inhibiting the transfer of P from ATP to glucose. This idea is in agreement with our findings, since the rise in ATP which occurs after glucose administration is due to an increased oxidation of glucose. If phlorhizin does inhibit the hexokinase reaction, then less glucose would be taken up by liver, and less ATP would be formed.

Kalckar (30) has reported the inhibition of anaerobic phosphorylation in kidney tissue by fluoride, however, no inhibition of aerobic phosphorylation was observed. Our animals injected with glucose and NaF showed a lower  $P^{32}$  content in the ATP than did the control glucose group. It is possible that fluoride partially prevents the further breakdown of hexose phosphates formed from the injected glucose and thereby the formation of ATP.

The manifold pathways by which oxidative energy may be derived accounts for the continuance of vital phenomena in the presence of various inhibitors. For example, malonate in the living animal may prevent the usage of some energy for aerobic phosphorylation, but other reactions proceed normally, and only a partial inhibition of energy processes (ATP formation) is observed.

It is of interest that no increase in hexose monophosphate concentration of the liver was observed following the administration of glucose. This suggests that glucose-6-phosphate, which is formed from glucose, undergoes a rapid reaction. The rise in ATP following the injection of glucose indicates that part of the glucose which is taken up by the liver is oxidized.

The regulation of liver phosphatase activity has received but little attention. It is well known that liver extracts hydrolyze glucose-6-phosphate to glucose and inorganic phosphate, the experiments reported here have demonstrated the rapid breakdown of glucose-6-phosphate in the living animal. Cori and his colleagues (4) have not been able to detect

any hexose esters in the aerobic phosphorylation of glucose or fructose except in the presence of fluoride. In the experiments of Colowick and Sutherland (31) glucose was converted to glycogen by purified enzymes only in the presence of barium ions. Therefore, in order for the intact liver to synthesize glycogen from glucose, regulating factors must be present which influence the activities of the enzymes phosphatase and phosphoglucomutase.

The specific site of the chemical action of insulin on the processes of intermediate metabolism has led to much speculation. Insulin is apparently not essential for the synthesis or breakdown of carbohydrates. Recent investigations have indicated that the site of insulin action is on the tricarboxylic acid cycle (16, 32, 33). Rice and Evans (33) have concluded that insulin is concerned in maintaining the functional integrity of either one or both of the enzyme systems involved in the reactions of fumaric and pyruvic acids or of oxalacetic and pyruvic acids. We have reached the conclusion that insulin action is concerned with the formation of energy-rich phosphate bonds. The evidence for this is discussed elsewhere (34).

We are indebted to Professor E. O. Lawrence and the staff of the Radiation Laboratory of the University of California for the  $P^{32}$  used in these investigations.

#### SUMMARY

1. Radioactive  $\text{Na}_2\text{HPO}_4$  injected into normal rats in a postabsorptive state is rapidly incorporated into the organic esters of liver. The uptake of  $P^{32}$  is greatest in the fraction which contains glucose-6-phosphate. The results also indicate that the turnover of glycerophosphate takes place at a relatively slow rate.

2. Glucose injection produces a significant increase in the radioactivity of the labile groups of adenosine triphosphate. The evidence suggests that only the labile groups of the adenosine triphosphate partake in phosphate transfer reactions in the intact animal. Glucose administration produces a general alteration in the phosphate cycle. The hexose monophosphate concentration, however, is not increased.

3. Insulin administration causes marked increases in the  $P^{32}$  contents of the total acid-soluble phosphate, the inorganic phosphate, and adenosine triphosphate of the liver. Insulin injected with glucose produces a greater increase in the  $P^{32}$  of the labile groups of adenosine triphosphate than does either one alone.

4. Injection of fluoride causes a marked increase in the  $P^{31}$  and  $P^{32}$  and the reducing values of the alcohol-insoluble fraction. This indicates that there is an accumulation of hexose-6-phosphate due to an inhibitory action on liver phosphatase. The inorganic  $P^{32}$  is lowered by fluoride. From

this it is concluded that some of the inorganic phosphate of the liver is derived from the breakdown of organic esters

5 After glucose injection, malonate and fluoride caused a partial inhibition of adenosine triphosphate formation. Phlorhizin exerted the greatest effect in inhibiting the formation of the organic pyrophosphate. Iodoacetate caused no interference in its production.

6 From the experimental results, the conclusion has been drawn that the phosphate cycle functions in the intact liver. The experimental results are discussed in relation to phosphorylation and respiratory processes in general.

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# STUDIES WITH RADIOACTIVE PHOSPHORUS OF THE CHANGES IN THE ACID-SOLUBLE PHOSPHATES IN THE LIVER COINCIDENT TO ALTERATIONS IN CARBOHYDRATE METABOLISM

## III THE EFFECT OF FASTING AND OF HIGH FAT, HIGH CARBOHYDRATE, AND HIGH PROTEIN DIETS

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The nature of the diet is known to influence the ability of the animal to handle administered glucose. Decreased glucose tolerance curves occur in animals that have been on a diet high in fat or protein (1, 2). It has also been found that fasting produces a decreased glucose tolerance, a phenomenon generally referred to as "hunger diabetes" (3, 4).

In Paper II of this series (5) it was shown that glucose administration causes striking changes in the phosphate cycle of the liver. Particularly noteworthy was the marked rise in the level of ATP (adenosine triphosphate).

There is a scarcity of information about the effects of different diets on intermediate metabolism. The relationship of the phosphate cycle of the liver to the diet was investigated in the experiments to be reported here. Evidence will be presented which demonstrates that decreased glucose tolerance is intimately related to an alteration of the phosphate cycle of the liver.

### EXPERIMENTAL

For the experiments on the effect of fasting, rats were deprived of food for a period of 72 hours. They were then injected intraperitoneally with trace doses of  $\text{Na}_2\text{H}^{32}\text{PO}_4$ . A second group of fasting rats was injected with 400 mg of glucose in addition to the radioactive phosphate. Both groups of animals were sacrificed 110 minutes after the administration of the  $\text{P}^{32}$ .

To study different dietary regimens, three groups of rats (ten in each group) were placed on a high fat, a high carbohydrate, and a high protein

\* The material of this paper was taken from a thesis submitted by N. O. Kaplan to the Graduate Division of the University of California in partial fulfillment of the requirements for the degree of Doctor of Philosophy, October, 1943.

diet respectively, for a period of 2 weeks<sup>1</sup>. The animals were then fasted for 12 hours and five members of each group were injected with  $\text{Na HP}^{32}\text{O}_4$  and five with 400 mg of glucose and the  $\text{Na}_2\text{HP}^{32}\text{O}_4$ . All animals were sacrificed 110 minutes after receiving the  $\text{P}^{32}$ .

The fractionation of the acid-soluble liver phosphates and the estimation of the phosphorus and the radioactivity of each fraction were carried out as described previously (5).

### Results

The influence of fasting and of the special diets on the phosphates of the liver are summarized in Tables I to III. The results of the different regimens are compared with those of control rats maintained on our stock colony diet.

*Effect of Fasting*—Fasting produces a decrease in the total acid-soluble  $\text{P}^{31}$ , but the total acid-soluble  $\text{P}^{32}$  is increased. These changes in  $\text{P}^{31}$  and  $\text{P}^{32}$  result in a considerably higher specific activity than occurs in the control group (Table II).

The greater part of the radioactivity in the fasting animals is in the form of inorganic phosphate. The increased inorganic  $\text{P}^{32}$  values in the livers of the fasted animals suggest that there is a decrease in phosphorylating reactions during fasting. This is further supported by the decrease found in the  $\text{P}^{32}$  of the various organic phosphorus components (alcohol precipitate, Hg precipitate, and residual phosphate).

Glucose administration to this group caused a less marked increase in the labile  $\text{P}^{31}$  and  $\text{P}^{32}$  of ATP than occurs in the control group (see Table III). Since glucose can be taken up by the tissues only after phosphorylation by ATP, this decrease in the ability to form ATP in the liver may be responsible, at least in part, for the decreased tolerance curves observed in fasting animals. The lowered level of ATP phosphorus observed in the fasting rats before the administration of glucose may also be a contributing factor to the decreased tolerance. Administration of glucose to the fasting animals produced no significant increase in the total acid-soluble  $\text{P}^{31}$  and  $\text{P}^{32}$  of the liver. The inorganic  $\text{P}^{32}$ , however, was decreased to about the same extent as in the control rats.

*High Carbohydrate Diet*—The high carbohydrate diet caused an increase in both the total acid-soluble  $\text{P}^{31}$  and  $\text{P}^{32}$ . This increase is largely to be

<sup>1</sup> The composition of the special diets (in percent) is as follows: High carbohydrate casein (commercial), 15, sucrose (powdered), 70, McCollum's Salt Mixture 185 (6), 5, yeast (brewers'), 5, cod liver oil, 5. High fat casein, 15, Salt Mixture 185, 5, yeast, 5, cod liver oil, 5, Crisco, 70. High protein casein, 84, Salt Mixture 185, 4, brewers' yeast, 8, cod liver oil, 4. To each kilo of diet there were added 200 gm of galen B extract and 10 gm of a sucrose filler containing riboflavin. Upon a food intake of 10 gm per day the rat would receive 2 gm of galen B and 40  $\gamma$  of riboflavin.

TABLE I

*Effect of Dietary State of Animal on Distribution of Acid-Soluble P<sub>1</sub> and P<sub>2</sub> in Liver*

All animals except those fasted 72 hours were fasted 12 hours before being injected with the Na<sub>2</sub>HPO<sub>4</sub> containing P<sub>2</sub>. All the animals were sacrificed 110 minutes after receiving the injection. The values for P<sub>1</sub> are in mg of P per 100 gm of fresh liver, P<sub>2</sub> in parts per 1000 of the administered dose per 100 gm of fresh liver. The measure of variability is the standard error of the mean.

		Fraction of insoluble barium salts						Fraction of soluble barium salts					
		Total acid soluble		Inorganic		Labile P of adenosine triphosphate		Residual		Mercury ppt		Alcohol ppt	
		p <sub>1</sub>	p <sub>2</sub>	p <sub>1</sub>	p <sub>2</sub>	p <sub>1</sub>	p <sub>2</sub>	p <sub>1</sub>	p <sub>2</sub>	p <sub>1</sub>	p <sub>2</sub>	p <sub>1</sub>	p <sub>2</sub>
Animals receiving only P <sub>2</sub>													
Controls (stock diet)	5	95.4	173.5	23.0	77.5	12.0	12.3	8.1	25.9	11.3	21.6	27.4	46.7
		±1.9	±6.4	±0.67	±3.5	±0.46	±0.77	±0.37	±2.3	±0.44	±1.3	±0.98	±2.1
Fasted 72 hrs	6	83.2	206.8	27.7	138.6	7.4	10.0	8.0	13.4	9.0	13.7	21.3	22.0
High carbohydrate	5	±2.6	±7.0	±0.94	±4.2	±0.27	±1.1	±0.33	±1.1	±0.33	±1.2	±0.88	±1.5
		107.2	212.7	19.9	66.0	13.2	15.1	10.5	30.3	12.4	28.7	31.4	53.9
" fat	5	±1.7	±4.5	±0.42	±2.9	±0.83	±1.3	±0.55	±1.5	±0.48	±2.0	±1.2	±2.9
" protein	5	80.6	143.2	22.9	88.6	5.7	10.1	8.8	18.1	8.8	15.3	21.9	26.5
		±2.9	±7.2	±0.66	±3.4	±0.51	±1.2	±0.46	±1.5	±0.55	±1.4	±0.67	±2.4
	5	86.0	204.4	30.6	119.6	8.6	10.7	9.5	25.2	8.8	20.1	21.8	30.6
		±0.79	±5.9	±0.60	±4.3	±0.23	±0.63	±0.65	±3.2	±0.50	±1.7	±1.1	±2.0
Animals receiving P <sub>2</sub> and 400 mg glucose													
Controls (stock diet)	5	103.4	206.6	20.0	58.3	20.8	46.5		13.4	11.2	21.0	29.7	27.9
Fasted 72 hrs	5	±2.0	±5.5	±0.72	±3.3	±0.45	±2.3		±1.2	±0.74	±0.80	±1.2	±2.1
		85.1	208.5	26.5	116.6	12.9	24.4			9.1	18.5	21.6	18.6
High carbohydrate	5	±1.8	±8.3	±1.0	±7.0	±0.75	±1.3			±0.39	±1.2	±0.96	±1.2
		115.4	241.3	19.4	58.6	22.3	57.7			13.5	33.0	34.4	32.5
" fat	1	±2.2	±6.8	±0.75	±2.3	±0.59	±2.5			±0.98	±1.5	±1.3	±2.1
		84.5	152.5	20.9	75.4	10.5	22.1			9.7	21.3	21.7	18.6
" protein	5	±2.0	±6.2	±0.64	±3.3	±0.52	±0.89			±0.87	±2.3	±0.67	±1.4
		87.9	218.9	27.0	101.4	14.5	30.3			9.0	25.9	22.2	27.5
		±1.2	±4.2	±1.2	±4.3	±0.62	±2.1			±0.71	±1.8	±0.86	±2.0

accounted for by increases in the alcohol-insoluble fraction and the residual fraction of insoluble barium salts. The inorganic  $P^{31}$  and  $P^{32}$  values are definitely decreased. This indicates that the animals on the high carbohydrate diet were better able to convert inorganic phosphate into the organic form than were the animals on the stock diet. Since the specific activities of most of the phosphate fractions were nearly the same in the group on the high carbohydrate diet and in the control group, the metabolic processes of the two groups were probably similar in kind, with the group on the high

TABLE II

Mean Specific and Relative Activities of Acid-Soluble Phosphate Fractions in Liver

Sa = specific activity =  $(P^1/P^{31}) \times 10$  of fraction R a = relative activity = of each fraction)/(total acid-soluble  $P^{31}) \times 10$

	Total acid soluble	Fraction of insoluble barium salts						Fraction of soluble barium salts					
		Inorganic		Labile P in adenosine triphosphate		Residual P		Hg ppt		Alcohol ppt			
		Sa	Ra	Sa	Ra	Sa	Ra	Sa	Ra	Sa	Ra		
Animals receiving only P <sup>32</sup>													
Stock diet (controls)	18 2	33 5	8 1	10 2	1 3	32 0	2 6	19 1	2 3	16 8	4 9		
Fasted 72 hrs	24 7	50 3	16 5	13 5	1 2	16 7	1 6	14 1	1 7	10 3	2 6		
High carbohydrate	18 2	33 2	6 1	11 5	1 4	28 9	2 8	23 0	2 7	17 4	5 0		
" fat	17 5	38 6	10 8	17 7	1 3	17 1	1 5	17 3	1 9	12 1	3 3		
" protein	24 2	39 0	14 0	12 5	1 2	26 6	2 9	22 9	2 3	14 0	3 6		
Animals receiving P <sup>32</sup> and 400 mg glucose													
Stock diet (controls)	20 0	29 3	5 6	22 2	4 5		1 3	18 7	2 0	9 5	2 7		
Fasted 72 hrs	24 4	44 0	13 7	19 0	2 9			20 4	2 2	8 6	2 2		
High carbohydrate	20 8	30 2	5 1	25 8	5 0			24 4	2 8	9 5	2 8		
" fat	18 1	36 2	9 0	21 0	2 6			22 0	2 5	8 6	2 2		
" protein	24 9	37 6	11 6	20 8	3 4			28 6	2 9	12 4	3 1		

carbohydrate diet possessing a higher reactive capacity for metabolizing sugar.

The rats on high carbohydrate diets responded to glucose administration in about the same manner as those on the stock diet. Slightly greater elevations of the ATP values occurred in this group. The increases in the total acid-soluble  $P^{31}$  and  $P^{32}$  which occur in the rats on high carbohydrate diets after administration of glucose are about the same as for the control group.

*High Fat Diet*—The livers of the rats on the high fat diet showed a decrease in the total acid-soluble  $P^{31}$ . This effect has also been observed by

Flock, Bollman, and Mann (7) on dogs. The total acid-soluble  $P^{32}$  also is decreased on the high fat diet and as a result the specific activity of the total acid-soluble phosphate is only slightly lower in the fat-fed than in the control animals (Table II).

The labile  $P^{31}$  in the ATP was considerably lower, while the  $P^{32}$  was not significantly lower in livers of the fat-fed than in the control rats. The  $P^{31}$  and  $P^{32}$  levels of the alcohol precipitate were decreased. The observed changes point to a decreased fixation of inorganic phosphate by the liver in animals on a high fat intake.

While administration of glucose to the fat-fed rats produced some increase in the ATP levels, the magnitude of the increase is much below that

TABLE III

*Mean Changes in  $P^{31}$  and  $P^{32}$  Induced by Administration of Glucose*

$P^{31}$  changes are in mg of P per 100 gm of fresh liver.  $P^{32}$  changes are in parts per 1000 of the administered dose per 100 gm of fresh liver.

	Total acid soluble		Fraction of insoluble barium salts						Fraction of soluble barium salts			
			Inorganic		Labile P in adenosine triphosphate		Residual		Hg ppt		Alcohol ppt	
	$P^{31}$	$P^{32}$	$P^{31}$	$P^{32}$	$P^{31}$	$P^{32}$	$P^{31}$	$P^{32}$	$P^{31}$	$P^{32}$	$P^{31}$	$P^{32}$
Stock diet	+8.0	+33.1	-3.0	-19.2	+8.8	+34.2		-16.5	-0.1	-0.6	+2.3	-18.9
Fasted 72 hrs	+1.9	+1.7	-1.2	-22.0	+5.4	+14.4			-0.8	+4.8	+0.3	-3.4
High carbohydrate	+8.2	+28.6	-0.5	-5.4	+9.1	+42.6			+1.1	+4.3	+3.0	-21.4
High fat	+3.9	+9.3	-2.0	-13.2	+4.8	+12.0			+0.9	+6.0	-0.2	-7.9
" protein	+1.3	+4.5	-3.6	-18.2	+5.9	+19.6			+0.2	+5.8	+0.4	-3.1

found in the rats on high carbohydrate diets and the control animals (Table III). No significant increase occurred in the total acid-soluble  $P^{31}$  and  $P^{32}$ , but there was a noticeable decrease in the inorganic  $P^{31}$  and  $P^{32}$  values following the injection of glucose into the fat-fed animals.

*High Protein Diet*—The distribution of the acid-soluble phosphates in the liver of the animals on the high protein diet was quite similar to that of the fasted animals. Characteristic of this is that there is a decrease in the total acid-soluble  $P^{31}$  and an increase in the total  $P^{32}$  in both conditions. The inorganic  $P^{31}$  and  $P^{32}$  were noticeably elevated. The specific activity of the P of the alcohol precipitate of the group on the high protein diet was lower than that of the control and those on high carbohydrate diets, however, it



was somewhat higher than that of the fasting and fat-fed animals (Table II)

The increase in the labile P of ATP following the administration of glucose is greater in the animals on the high protein diet than in the fasting or fat-fed animals, but the increase is less than in the animals fed the stock or high carbohydrate diets

Glucose administration did not significantly alter the total acid-soluble  $P^{31}$  and  $P^{32}$  in the animals on high protein diets. The inorganic  $P^{31}$  and  $P^{32}$  decreased to about the same extent as in the animals on the stock diet. Only a slight drop in the  $P^{32}$  of the alcohol precipitate resulted from the injection of glucose, this is quite striking in the light of the considerable reduction that occurs in the high carbohydrate-fed and control rats (Table III)

### DISCUSSION

The results reported here illustrate the great influence of diet on the acid-soluble phosphates of the liver

The decreased ability to form ATP after glucose administration is of great significance to the etiology of the reduced glucose tolerance curves found in fasting animals and in animals fed high fat and high protein diets. The results of the studies on phosphorylation reactions make it appear highly probable that glucose can be utilized by tissues only after first being phosphorylated by the adenylic acid system (8). The decreased levels of ATP found in the livers of the high fat- and high protein-fed and of the fasting rats suggest a decreased capacity to phosphorylate glucose and, consequently, a decrease in the glucose tolerance.

It is of interest that the higher the existing level of labile  $P^{31}$  in ATP is before the administration of glucose, the greater is the rise in the labile  $P^{31}$  following the administration of glucose. The interpretation of this occurrence is that part of the glucose taken up by the liver is oxidized and the resulting energy converted into  $\sim\text{ph}$ ,<sup>2</sup> which facilitates further utilization of the sugar.

The degree of increase in ATP which follows the administration of glucose to the animals on the various regimens takes place in the following decreasing order: (1) high carbohydrate diet, (2) stock colony diet, (3) high protein diet, (4) fasted 72 hours, and (5) high fat diet. The magnitude of the increase in the ATP of the liver bears a direct relationship to the degree of glucose tolerance on each of the regimens. It is known that a high carbohydrate diet produces an increase in glucose tolerance, whereas a high fat diet produces a marked decrease in glucose tolerance. Animals on high

<sup>2</sup> The symbol  $\sim\text{ph}$  represents a high energy phosphate bond as defined by Lipmann (9)

protein diets have tolerance curves which are intermediate between those of carbohydrate- and fat-fed animals (10)

A marked increase in the total acid-soluble  $P^{31}$  and  $P^{32}$  following the introduction of glucose occurred only in the group on the high carbohydrate diet and in the control group. We believe that this increase fits in with the view-point that glucose stimulates the secretion of insulin. In a previous work it was observed that insulin produces a marked elevation in the total acid-soluble  $P^{31}$  and  $P^{32}$  of the liver and an accelerated disappearance of  $P^{32}$  from the blood (11). Furthermore, it is known that the decrease in serum inorganic phosphate which follows the administration of glucose does not take place in depancreatized animals (12).

The fact that some rise occurs in the ATP levels of the fat-fed, protein-fed, and the fasted rats may be considered as evidence that glucose is utilized by the liver even when the insulin content is low. This is further suggested by the significant decrease in inorganic  $P^{31}$  and  $P^{32}$  following glucose administration in the above dietary states.

The lowering of the total ATP in the fasted and protein-fed animals is probably indicative of a decrease in oxidative reactions. Whether the extremely low ATP levels in the fat-fed animals also indicates a lowering of oxidative processes is not clear. The low ATP content may perhaps be explained by the following observations. (1) The work of Muñoz and Leloir (13) is open to the interpretation that the oxidation of fatty acids to hydroxy acids by liver enzymes requires energy which is probably supplied by the  $\sim$ ph present in ATP.<sup>3</sup> (2) Stadie (15) states that the liver utilizes only a negligible amount of the ketone substances which it produces. Consequently on a high fat diet the liver would be appropriating  $\sim$ ph for the formation of ketone substances and would not be gaining any fresh  $\sim$ ph from the oxidation of these compounds. This would lead to a decrease in the  $\sim$ ph reservoir (ATP of the liver).

The values of the labile  $P^{32}$  of the ATP were quite similar in all of the different groups prior to their receiving glucose. Since all the animals were fasted for at least 12 hours, this fact may indicate that the oxidative reactions that occur during fasting are quite similar regardless of the previous dietary history. The similar increase in the total acid-soluble and inorganic  $P^{32}$  in the livers of both the protein-fed and fasting animals indicates a similarity in the metabolic reactions in these conditions. This is readily understandable, since the proteins of the body are heavily drawn upon during fasting.

The decrease noted in the  $P^{31}$  and  $P^{32}$  values of the alcohol precipitate in

<sup>3</sup> Recently Lehninger (14) has shown that octanoic acid oxidation requires the presence of ATP. This is further evidence that oxidation of fatty acids in liver necessitates the presence of  $\sim$ pH bonds.

the fasting animals and those receiving the high fat and high protein diets seems to be due to a decline in the glycerophosphate concentration. The relation of glycerophosphate to carbohydrate metabolism is still obscure.

We are greatly indebted to Professor E. O. Lawrence and the staff of the Radiation Laboratory of the University of California for supplying us with the radioactive phosphorus used in this investigation.

#### SUMMARY

1 The influence of fasting and of high fat, high protein, and high carbohydrate diets on the distribution and turnover of the acid-soluble phosphate components of the liver was studied by means of tracer experiments with  $P^{32}$ .

2 The total acid-soluble  $P^{31}$  content of the liver decreased in the fasted and the high fat- and high protein-fed animals. The total acid-soluble  $P^{31}$  is increased by a high carbohydrate intake. The total acid-soluble  $P^{31}$  content of the liver is increased on all but the high fat regimen.

3 In the fasted, fat-fed, and protein-fed rats a great deal of the  $P^{32}$  is present in the inorganic form. These animals show a decreased ability to fix inorganic phosphate. The high carbohydrate-fed animals show a decrease in the inorganic  $P^{32}$ . They convert a larger percentage of the labeled P into organic form than do the animals on any other regimen.

4 Decreased values for the labile  $P^{31}$  of adenosine triphosphate are found in the livers of the fasting, protein-fed, and fat-fed animals. The decrease is most marked in the fat-fed animals. Little difference is found in the labile  $P^{32}$  of adenosine triphosphate of all the different groups.

5 The capacity to form adenosine triphosphate after glucose administration on the different regimens was found to decrease in the following order: (1) high carbohydrate diet, (2) stock diet, (3) high protein diet, (4) fasted 72 hours, (5) high fat diet. It is suggested that the adenosine triphosphate content of the liver is of primary importance in determining the shape of the glucose tolerance curve. The decreased glucose tolerance curve on the above regimens runs parallel with the decreasing adenosine triphosphate content of the liver.

6 Increases in total acid-soluble  $P^{31}$  and  $P^{32}$  following administration of glucose occurred only in the animals fed carbohydrate. A decrease in inorganic  $P^{32}$  occurred in all groups as the result of glucose administration.

7 A decrease in the  $P^{31}$  and  $P^{32}$  content of the phosphate fraction consisting of glycerophosphate and hexose monophosphates occurs in the fasting animals and in those on the high protein and high fat diets.

8 The facts cited above are discussed in relation to the effect of the dietary state on insulin secretion and carbohydrate metabolism.

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# THE ACTION OF INSULIN ON THE PHOSPHATE CYCLE

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The specific site of the chemical action of insulin has been the subject of much speculation. Insulin is apparently not essential for the synthesis or breakdown of carbohydrates. There is no positive evidence to indicate that insulin has any effect on the reactions of the Embden, Meyerhof, Parnas, and Cori schemes.

Recent investigations have suggested that the action of insulin may be on the tricarboxylic acid cycle. Krebs and Eggleston (1) first demonstrated that insulin causes an increased oxidation of certain members of the tricarboxylic acid cycle in pigeon breast muscle. Although Shorr and Barker (2) failed to observe any influence of insulin in preparations of rabbit, cat, and dog muscle, the effect on pigeon breast muscle has been confirmed by others (3-5). Stare and Baumann (5) observed that while excised rabbit heart and skeletal muscle, and chicken breast muscle, are quantitatively less spectacular in their response to insulin, they respond qualitatively in the same manner as the more rapidly respiring pigeon breast muscle.

In the course of the study of insulin action, Stare and Baumann (3) observed that injection of malonate in the intact animal prevented the hypoglycemic action of insulin. They also noted a temporary rise in blood sugar when malonate alone was administered.

Rice and Evans (6) have reported that insulin induces an increased oxidation of pyruvate which could be inhibited by malonate. These authors found that insulin had no effect on the oxidation of citrate and of  $\alpha$ -ketoglutarate. From these investigations it appears that insulin may have a catalytic effect on certain of the reactions of the Krebs' tricarboxylic acid cycle.

Since oxidation through the tricarboxylic acid system results in the formation of  $\sim$ pH bonds<sup>1</sup> (8, 9), it is plausible to expect that malonate would influence the rise in liver ATP (adenosine triphosphate) which follows the administration of insulin. The experiments reported below were under-

\* The material in this paper was taken from a thesis submitted by N. O. Kaplan to the Graduate Division of the University of California in partial fulfillment of the requirements for the degree of Doctor of Philosophy, October, 1943.

<sup>1</sup> The symbol  $\sim$ pH represents a high energy phosphate bond as defined by Lipmann (7).

taken to obtain evidence on the relation between insulin and the tricarboxylic acid cycle

*Inhibition of Insulin Action by Malonate in Vivo*—For these experiments there were used three groups of five adult rats each, fasted for 12 hours. One group injected with 1 ml of saline solution served as the control. A second group received 4 units of insulin and a third group 1 ml of M malonate solution in addition to 4 units of insulin. All administrations were by intraperitoneal injection. The animals were sacrificed 75 minutes after treatment. The results are reported in Table I.

The data show that there is a statistically significant inhibition by malonate of the rise in ATP which is induced by insulin.<sup>2</sup> The lower ATP values in the malonate-injected animals can be associated with an increase in inorganic phosphate. The rise in inorganic phosphate indicates that malonate

TABLE I

*Inhibition of Insulin Action on Liver Phosphates by Malonate*

All values are given in mg of P per 100 gm of fresh liver. Each group consisted of five animals. The measure of variability is the standard error.

	Total acid soluble P	Inorganic P	Labile P in adenosine triphosphate	Rest P
Controls, 1 ml saline	95.5 ± 1.9	22.8 ± 1.0	13.6 ± 0.8	58.6 ± 1.2
4 units insulin	109.4 ± 4.5	27.3 ± 0.9	17.8 ± 1.1	64.3 ± 2.8
4 " " + 1 ml 1 M malonate	108.7 ± 2.7	30.6 ± 1.1	15.0 ± 0.6	63.1 ± 2.2

\* Rest P represents the difference between the total acid-soluble P and inorganic P + labile P of adenosine triphosphate.

ate prevents the uptake of inorganic phosphate. Malonate has no effect on the increase in the total acid-soluble phosphate following the injection of insulin. It may be inferred from the data that AMP is formed from reactions involving the tricarboxylic acid cycle in the intact liver. The results indicate an influence of insulin on the tricarboxylic acid cycle in the living animal.

*Effect of Insulin and Malonate on Liver Phosphates of Rats on High Fat Diet*—Insulin improves the glucose tolerance of animals on a high fat diet (10). Therefore it appeared desirable to determine the influence of insulin on the liver phosphates of animals receiving a high fat diet.

<sup>2</sup> Whether larger doses or the continuous administration of malonate would cause a more marked effect has yet to be determined. It is interesting to note that Stare and Baumann found that malonate hindered the hypoglycemic action of insulin in the rabbit for a period of only 1 hour.

In this series of experiments the rats had free access to the high fat ration (11) up to the time of injection. The treatment was the same as for the previous series. The results are summarized in Table II.

The data show that insulin produces a small increase in the total acid-soluble phosphate and a very striking increase in the ATP content of the rats fed a high fat diet. The marked change in inorganic phosphate which occurs after administration of insulin to carbohydrate-fed animals is absent in the fat-fed animals.

The influence of malonate on the fat-fed animals is similar to its effect on those on a carbohydrate diet, namely, there is a smaller increase in ATP and an increase in inorganic phosphate.

TABLE II

*Effect of Insulin and Malonate Administration on Livers of Animals Fed High Fat Diet*

All values are given in mg of P per 100 gm of fresh liver. Each group consisted of five animals. The measure of variability is the standard error.

	Total acid soluble P	Inorganic P	Labile P in adenosine triphosphate	Rest P*
Controls	79.2 ± 1.7	22.9 ± 0.8	5.8 ± 0.4	50.4 ± 1.2
Injected with 5 units insulin	84.7 ± 1.2	22.1 ± 0.5	10.0 ± 0.4	52.8 ± 1.3
5 units insulin + 1 ml 1 M malonate	84.9 ± 1.9	26.0 ± 0.8	7.7 ± 0.7	51.2 ± 0.8

\* Rest P represents the difference between the total acid-soluble P and inorganic P + labile P of adenosine triphosphate.

The results on the high fat diet suggest that insulin may influence the oxidation of fat by directing the breakdown products of fatty acids (ketone substances) into the tricarboxylic acid cycle. Breusch (12) has recently reported on the occurrence of an enzyme named citrogenase, which catalyzes the reaction, acetoacetic acid + oxalacetic acid → citric acid + acetic acid. If insulin is found to influence this enzyme, the implications will indeed be great.

In connection with the possible action of insulin on fat metabolism, it is interesting to note that Dick *et al* (13) have found that insulin decreases the severity of hunger ketosis. The results of Table II are also supported by the observation of Szent-Gyorgi and coworkers (14) that malonate increased the urinary excretion of ketone bodies for a period of about 2 hours.

In fat-fed animals treated with insulin the ability to synthesize ATP after the administration of glucose is still further increased. The results of such a series of experiments are given in Table III. In this test the rats fed high



fat diets were injected subcutaneously daily with 4 units of insulin for 5 days before the test. On the day of the test they are fasted for 12 hours and then injected intraperitoneally with 400 mg of glucose.

The fact that the fat-fed animals respond to the insulin indicates a lack of insulin secretion in this condition. Chambers (15) reports that insulin administration for several days to fasting dogs improves the glucose tolerance of these animals. This agrees with the results given in Table III.

*Influence of Insulin on Fate of Administered Lactate*—Cori and Cori (16) have observed that administered lactate is largely converted into liver glycogen. Flock, Bollman, and Mann (17) found a normal utilization of pyruvate and lactate in depancreatized dogs. More recently, Bueding *et al* (18) noted that insulin does not influence the rate of disappearance of administered pyruvate from blood.

TABLE III

*Effect of Insulin Administration on Glucose Tolerance of Fat-Fed Animals As Indicated by Liver Phosphate Changes*

All values are given in mg of P per 100 gm of fresh liver. The animals that were given insulin were injected daily with 4 units of the hormone subcutaneously 5 days prior to the administration of glucose. Both groups of four animals were fasted for 12 hours before the 400 mg of glucose were administered. The animals were sacrificed 90 minutes after receiving the glucose. No insulin was given on the day of  $P^{32}$  injection. The measure of variability is the standard error.

	Total acid soluble P	Inorganic P	Labile P in adenosine triphosphate	Rest P
Fat-fed controls	82.7 $\pm$ 1.9	20.5 $\pm$ 0.8	11.3 $\pm$ 0.7	50.9 $\pm$ 0.9
" + insulin	89.2 $\pm$ 1.2	20.0 $\pm$ 0.6	14.7 $\pm$ 0.8	54.7 $\pm$ 1.0

The present experiments were undertaken to determine whether insulin influenced the distribution of the liver phosphates upon introduction of lactate. The results indicate that insulin has a marked effect on the disposition of the three carbon carbohydrate intermediates in the liver.

The experiments were carried out on normal rats fasted for 20 hours. One group received 400 mg of sodium lactate (5 per cent solution) by stomach tube, a second group the sodium lactate and 4 units of insulin, and a third group (controls) only 0.9 per cent sodium chloride solution. The results are summarized in Table IV.

From Table IV it is evident that insulin prevents the formation of liver glycogen from lactate. The marked changes in the acid-soluble phosphate indicate that insulin stimulates the oxidation of lactate. This is understandable, since the increase in organophosphates could have resulted only from an acceleration of oxidation processes.

Lactate alone produced a small but significant increase in ATP. This is an indication that some lactate is normally oxidized in the liver.

The results with lactate suggest that the fate of the three carbon compounds in the liver is subject to hormonal control, and that insulin may influence metabolism by the stimulation of the aerobic oxidation of pyruvic acid with formation of a large number of energy-rich phosphate bonds.

TABLE IV

*Influence of Insulin and Lactic Acid on Liver Phosphates*

All values are given in mg of P per 100 gm of fresh liver. The figures given are the means and their standard errors. Each group consisted of eight animals.

	Total acid soluble P	Inorganic P	Labile P in adenosine triphosphate	Rest P	Liver glycogen*
					<i>per cent</i>
Controls	94.4 ± 1.5	25.4 ± 0.78	11.3 ± 0.67	58.8 ± 1.4	0.199 ± 0.022
400 mg lactic acid	94.7 ± 1.8	23.3 ± 0.48	14.9 ± 0.41	56.9 ± 1.9	0.684 ± 0.101
400 mg lactic acid + 4 units insulin	131.0 ± 2.4	30.7 ± 0.69	20.9 ± 0.68	79.5 ± 2.1	0.200 ± 0.016

\* We are greatly indebted to Mr. Virgil V. Herring of the Institute of Experimental Biology for the glycogen determinations.

## SUMMARY

1. Malonate inhibits the degree of increase in liver adenosine triphosphate which follows the injection of insulin; coincident with the lower adenosine triphosphate values, there is an increase in inorganic phosphate.

2. Insulin increases the adenosine triphosphate level in the liver of animals on a high fat diet; malonate inhibits this increase. Insulin given to fat-fed animals increases their ability to form adenosine triphosphate when glucose is administered.

3. Insulin prevents the formation of liver glycogen from simultaneously administered lactate. Lactate given alone causes a slight increase in adenosine triphosphate. The administration of lactate and insulin together results in marked changes in the acid-soluble phosphate fractions of the liver.

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# THE RÔLE OF BICARBONATE IN THE ACTION OF SERUM IN SUPPORTING TISSUE RESPIRATION\*

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Most tissues exhibit higher and more constant rates of respiration *in vitro* when suspended in serum rather than in Ringer's solution (2, 3, 9, 10, 13-15, 17, 20, 22, 24, 25). Canzaneli *et al* (3) found this effect to be due largely to substances present in the ultrafiltrate of serum, although smaller effects were exerted by serum proteins as well. In attempting further to fractionate serum ultrafiltrate for the purpose of identifying some of these metabolically active constituents, we have found that a considerable and perhaps major part of its activity in supporting tissue respiration is due to its bicarbonate content. These results are reported in the following communication.

## Methods

Tissue respiration in serum may be measured in two ways, differing in the management of its bicarbonate content. If this is to be maintained at its original level, the gas phase must contain 5 per cent  $\text{CO}_2$ , thus precluding the absorption of  $\text{CO}_2$  by alkali as in simple Warburg manometry. Either the indirect Warburg two vessel method or one of the direct differential methods may be employed (6). The other type of procedure is to treat the serum with acid and evacuate it to remove bicarbonate (2, 9, 14, 22). The pH is then restored to 7.4 with alkali, and additional buffer may be added if desired. Such material is called "neutralized" serum, the essential feature being that the pH is maintained at a physiological level in the absence of  $\text{CO}_2$  in the gas phase. It may accordingly be used in simple Warburg manometry in which respiratory  $\text{CO}_2$  is absorbed by alkali throughout the experiment.

The stimulating<sup>1</sup> effect of serum has been demonstrated by each of these methods and it has been found by the writer in earlier experiments (22).

\* This work was aided by a grant from the John and Mary R. Markle Foundation.

<sup>1</sup> The higher rate of respiration observed when tissue is suspended in serum, serum ultrafiltrate, or Ringer bicarbonate solution, in comparison with that in Ringer-phosphate solution, is referred to throughout this paper as the stimulating effect of these media. The intent, however, is to attribute to these solutions an ability to sustain tissue respiration *in vitro* that is superior to that of Ringer phosphate solution.

that the respiration of rabbit bone marrow is actually the same in unaltered serum (differential method) and in neutralized serum (simple Warburg method), in each instance averaging about 70 per cent higher than in Ringer's solution. This indicated that the presence or absence of  $\text{CO}_2$  in the gas phase was of little if any consequence, and the same inference might reasonably be extended to apply to the presence or absence of bicarbonate in the medium. That this last inference would be incorrect, however, will be demonstrated by the experiments to be reported.

Standard methods for measuring tissue respiration have been employed. In the experiments requiring  $\text{CO}_2$  in the gas phase, Summerson differential manometers (19) have been used, in the others simple Warburg manometers. All measurements were made in duplicate or triplicate. The experimental period varied from 1 to 3 hours, depending upon the activity of the tissue, the temperature of the water bath was maintained at  $38^\circ$ . Tissue slices were prepared by hand. Bone marrow was stripped from the blood vessels and teased into small pieces with a pair of forceps while immersed in a Petri dish containing Ringer's solution. The dish was tilted to provide a "beach" on which the teasing could be carried out readily. This method is much simpler and yields just as satisfactory results as the slicing technique described earlier (21). After blotting on filter paper, the samples of tissue were weighed on a torsion balance. Dry weights were usually not determined, since we have been concerned largely with relative rather than absolute  $Q_{\text{O}_2}$  values. The animals employed were fed *ad libitum*, and were killed by a blow on the head. A Beckman pH meter was used for determining pH. The Ringer solutions had the composition previously employed (22) but the final concentration of phosphate buffer (pH 7.4) was increased from  $\text{M}/150$  to  $\text{M}/40$ . All solutions contained 200 mg per cent of added glucose.

*Ringer-Bicarbonate Versus Ringer-Phosphate Solution*.—In these experiments, the  $Q_{\text{O}_2}$  of tissues suspended in Ringer-phosphate solution was compared with that of the same tissues in Ringer-bicarbonate medium with 5 per cent  $\text{CO}_2$  in the gas phase. The bicarbonate concentration was 25 mM per liter, the same as that of most normal sera. The results (Table I) show that, of the tissues studied, all except rabbit kidney show an appreciably higher rate of respiration in the bicarbonate- $\text{CO}_2$  medium. There is a large variation in the magnitude of the effect, however, noted with samples of the same tissue taken from different animals of the same species, perhaps accounting for varied statements on this point in the literature (*cf* (5) versus (13)). The three zeros recorded in the last column, however, are in each case isolated instances, and there is little doubt that the usual result to be expected is an appreciably enhanced rate of respiration in bicarbonate- $\text{CO}_2$  medium. The reason for the variability remains

to be elucidated. Rabbit bone marrow appears to show a relatively large and consistent effect and has been used throughout most of the following experiments. The failure of rabbit kidney to show this effect apparently represents a true metabolic characteristic of this organ of a herbivorous animal, but we are in ignorance of the basis on which it rests. Control experiments indicate that the differences in rate of respiration in the two media are not attributable to the presence or absence of phosphate buffer or to differences in calcium ion concentration.

*Partially Neutralized Ringer-Bicarbonate Medium*—The effect of bicarbonate just described may also be observed in the absence of  $\text{CO}_2$  in the gas phase. When one neutralizes Ringer-bicarbonate solution by the method we have described for neutralizing serum (22) and employs simple

TABLE I  
*Increase in Tissue Respiration in Ringer-Bicarbonate- $\text{CO}_2$  Medium in Comparison with That in Ringer-Phosphate Medium*

Tissue	No of experiments	Average increase in respiration in bicarbonate $\text{CO}_2$	Range
		per cent	per cent
Rabbit bone marrow	33	41	0 to 95
Guinea pig liver	6	20	0 " 49
Rat liver*	4	20	0 " 52
" " †	11	43	-9 " +112
Rabbit kidney cortex	4	-8	-4 " -15
Rat kidney cortex	2	19	10 " 28

\* Our experiments

† Previously unreported data kindly provided by Dr William H. Summerson of the Department of Biochemistry

Warburg manometry with absorption of  $\text{CO}_2$  by alkali, the medium still shows a stimulating effect on respiration which is nearly as great as that found when the original bicarbonate concentration is maintained and the measurement is made in differential manometers with 5 per cent  $\text{CO}_2$  in the gas phase (Table II). This rather surprising result is the observation which prompted the present study. It suggested either that the salt formed in neutralization stimulates respiration or that the neutralization is incomplete, effective amounts of bicarbonate remaining in solution even in the absence of  $\text{CO}_2$  in the gas phase. By control experiments with added  $\text{NaCl}$ , the latter explanation was found to be the correct one, and a simple calculation revealed that the amounts of acid we had been using for neutralization were in fact sufficient to neutralize only about half the bicarbonate present. The amount remaining has a dual effect, it acts as

an alkali reserve for the neutralization of acid formed by aerobic glycolysis and it has a separate specific effect on respiration. It is obviously important to separate these two effects and to determine (1) how much bicarbonate remains in solution when it is exposed to alkali in the respirometer vessels and (2) the relationship between the concentration of bicarbonate and its effect upon respiration. These points are dealt with in the following section.

*Bicarbonate Effect in Simple Warburg Manometry*—When one adds increments of bicarbonate to Ringer-phosphate medium, the phosphate acts as an acid and displaces some  $\text{CO}_2$  from the medium even before it is placed in the respirometer vessels. Thereupon, further  $\text{CO}_2$  is absorbed from the medium by the alkali in the center wells of the vessels and the bicarbonate content of the medium is reduced still further, rapidly at first and more

TABLE II  
*Rabbit Bone Marrow Respiration in Partially Neutralized Versus Unneutralized Bicarbonate Medium*

Experiment No	Increase in respiration in	
	Partially neutralized bicarbonate	Bicarbonate with 5 per cent $\text{CO}_2$
	<i>per cent</i>	<i>per cent</i>
58	60	48
60	59	69
61	55	35
62	61	95
63	41	65
Average	55	62

slowly as the experiment continues. The amount present at any time may be determined by transferring the medium to a vessel not containing alkali, tipping in acid from the side well, and measuring the pressure increase due to liberation of  $\text{CO}_2$  from the bicarbonate. In the experiments now to be considered, this procedure has been followed and in addition the effect of the medium on the respiration of bone marrow was determined. In separate duplicate vessels, 5, 10, and 15 mm per liter of bicarbonate were added to the Ringer-phosphate medium and the rates of respiration of the marrow in these solutions were compared with that in control Ringer-phosphate solution. Samples of these solutions without tissue were also placed in similar vessels and at the middle of the experimental period their content of  $\text{CO}_2$  was determined by the method just described. The results of two typical experiments are shown in Fig 1. From this it is clear that small amounts of bicarbonate remain in the medium when it is

exposed to alkali and that only about 3 mm per liter need to be present in order to obtain maximal stimulating effects

In the solutions containing bicarbonate, the pH is slightly higher than in the control Ringer-phosphate solution. The results shown in Fig 1 have been corrected for this pH effect by means of a pH- $Q_{O_2}$  curve. Over the range encountered (pH 7 to 7.6) this correction was within 10 per cent of the observed  $Q_{O_2}$ .

In the experiment shown in Fig 2, increments of bicarbonate were added to completely  $CO_2$  free serum,<sup>2</sup> rather than to Ringer's solution, and the  $CO_2$  content of the medium was determined both at the beginning and at the end of the experiment. Also, the media so tested were taken from vessels containing tissue, the tissue being first removed by centrifugation

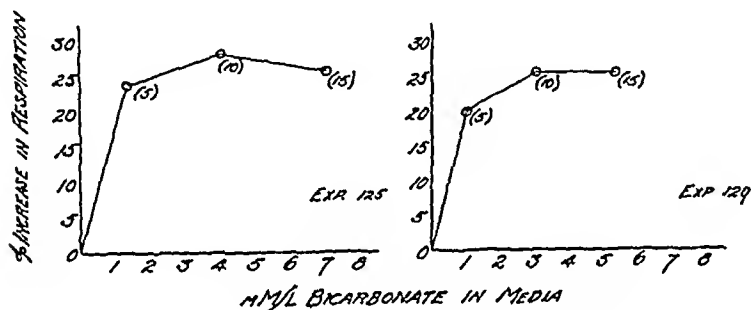


Fig 1 Effect on marrow respiration of adding increments of bicarbonate to Ringer-phosphate medium. The figures in parentheses indicate mm per liter of bicarbonate originally added to the medium.

Fig 2 shows that the  $CO_2$ -free serum alone augmented respiration by 25 per cent and also that increments of added bicarbonate brought about still further increases in the rate of respiration. The  $CO_2$  content of the medium fell during the 2 hour duration of the experiment, but the rate of respiration was not affected by this decrease in bicarbonate content. In this particular experiment a small amount of bicarbonate was still present at the end of the experiment, but in others the bicarbonate content fell to zero during the experimental period, still without affecting the augmented rate of respiration. The inference is that a small amount of bicarbonate at the beginning of the experiment permits the elaboration

<sup>2</sup>  $CO_2$  free serum is readily prepared by bringing the pH below 5.5 with HCl (about 30 mm of HCl per liter) and placing it in a flat covered dish containing KOH in an inset. The dish may either be shaken for 30 minutes at room temperature or allowed to stand overnight in a refrigerator. Before use, the serum is brought to pH 7.4 with alkali and buffered there with M/40 (final concentration) phosphate buffer.



within the tissue of some metabolically active substance, possibly oxalacetate (11), which enables respiration to continue at an augmented rate even if all the bicarbonate is subsequently removed. This, at least, is a working hypothesis to explain the results, further studies will be required to elucidate the precise mechanism of the bicarbonate effect. In any case, the results in this section show that only small amounts of bicarbonate need to be present in order to exert an effect and that these amounts may persist for some time after buffered bicarbonate-containing medium is exposed to alkali, a circumstance which permits the bicarbonate effect to be observed in simple Warburg manometry with virtual absence of  $\text{CO}_2$  in the gas phase.

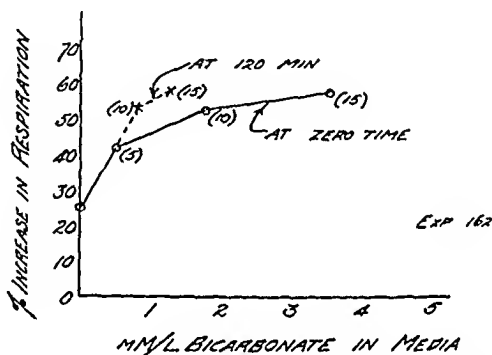


FIG 2 Effect on marrow respiration of adding increments of bicarbonate to  $\text{CO}_2$ -free serum. The figures in parentheses indicate mm per liter of bicarbonate originally added to the medium.

*Summation of Bicarbonate and Serum Effects*—The evidence just presented (Fig 2) shows that in addition to the effect of bicarbonate there is a separate stimulating effect of  $\text{CO}_2$ -free serum which, in the interests of simplicity, we shall call the serum effect, while appreciating that in all probability it is a composite effect of the action of various constituents. The question now to be considered is the extent to which the rate of respiration measured in serum equilibrated with 5 per cent  $\text{CO}_2$  (differential method) is a summation of the serum and bicarbonate effects. The pertinent data are shown in Table III. The serum and bicarbonate effects are listed in Columns 1 and 2, and their arithmetic sum is given in the last column. When this is compared with the effect of serum- $\text{CO}_2$  (Column 3), it is evident that the combined effect (Column 3) may be somewhat greater or somewhat less than the sum of the separate effects (last column), but on the average the extent of summation is within the limits of experimental

error One also notes that the serum effect is usually somewhat larger than the bicarbonate effect

TABLE III

*Comparison of Effect of CO<sub>2</sub>-Free Serum with That of Ringer-Bicarbonate Solution and Serum (Last Two with 5 Per Cent CO<sub>2</sub>)*

Experiment No	Rabbit bone marrow			(1) + (2)
	Increase in respiration in			
	CO <sub>2</sub> -free serum (1)	Ringer bicar bonate CO <sub>2</sub> (2)	Serum CO <sub>2</sub> (3)	
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	
137	51	26	81	77
141	35	35	52	70
142	37	37	91	74
158	48	35	55	83
163	53	22	75	75
164	33	30	50	63
165	19	3	19	22
Average	39	27	60	66

TABLE IV

*Comparison of Effect of Serum Ultrafiltrate with That of Bicarbonate Solution (Both with 5 Per Cent CO<sub>2</sub>)*

Experiment No	Rabbit bone marrow Increase in respiration in	
	Bicarbonate solution	Serum ultrafiltrate
	<i>per cent</i>	<i>per cent</i>
110	40	42
113	64	74
114	44	31
115	47	110
117	57	79
146	24	20
151	64	70
157	37	56
Average	47	60

*Bicarbonate Effect in Serum Ultrafiltrates*—Ultrafiltrates of human and horse serum were prepared by filtration through Visking membranes under 30 pounds per sq in of pressure The activity of the ultrafiltrates in stimulating tissue respiration appeared to be independent of the species

difference and was not altered by inactivating the serum. Table IV shows that, in the case of rabbit bone marrow, the stimulating effect of serum ultrafiltrate is only slightly greater on the average than that of Ringer-bicarbonate solution when both measurements are made in the presence of 5 per cent  $\text{CO}_2$ . This suggests that most of the activity of such ultrafiltrates is due to their bicarbonate content. However, a clearer picture of the true state of affairs may be obtained by reference to Table V. Here it is evident that in the case of rabbit bone marrow,  $\text{CO}_2$ -free ultrafiltrate (prepared in the same way as  $\text{CO}_2$ -free serum) shows a definite stimulating

TABLE V  
*Bicarbonate Effect in Serum Ultrafiltrates*

	Experiment No	Increase in respiration in			(1) + (2)
		$\text{CO}_2$ -free ultrafiltrate (1)	Ringer $\text{HCO}_3$ with 5% $\text{CO}_2$ (2)	Ultrafiltrate with 5% $\text{CO}_2$ (3)	
		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	
Rabbit bone marrow	117	41	57	79	98
	146	13	24	20	37
	151	19	64	70	83
	157	30	37	56	67
	181	19	31	31	50
Average		24	43	51	67
Guinea pig liver	149	23	0	57	23
	155	40	12	62	52
	167	8	27	48	35
	168	14	32	42	46
	174	44	12	37	56
	175	65	38	104	103
	177	10	13	40	23
	178	66	27	72	93
Average		34	20	58	54

effect. It is smaller, however, than the bicarbonate effect, and the two effects show incomplete summation, thus accounting for the results of Table IV. With guinea pig liver, the variation in individual experiments is greater, but the average result differs from that obtained with rabbit bone marrow in that the ultrafiltrate effect is greater than the bicarbonate effect and the two effects are approximately additive.

#### DISCUSSION

The evidence presented in this paper indicates that bicarbonate is a metabolically active substance, in the sense that in its presence the respira-

tion of certain tissues is maintained at a higher level than in Ringer-phosphate solution. This is a confirmation and extension of scattered reports of other investigators (1, 4, 12, 13) which seem not to have received proper attention in view of the original negative, and perhaps exceptional, results of Dickens and Simer (5). Also, the metabolic rôle of bicarbonate and  $\text{CO}_2$  has recently been brought to light in biochemical studies with carbon isotopes (7, 8, 11, 18, 23). Whether the intermediary metabolic pathways proposed by these authors apply to the various tissues studied in this report and what rôle, if any, is played by carbonic anhydrase, remain subjects for further investigation.

Apart from the mechanism of the action of bicarbonate, the fact that its presence or absence may influence respiration must be appreciated in experiments designed to elucidate the nature of the other metabolically active substances in serum or its ultrafiltrate. Of practical importance in this connection is the hitherto unappreciated fact that the use of neutralized serum or its ultrafiltrate in simple Warburg manometry does not preclude an effect of bicarbonate, unless special precautions are taken to insure its complete removal from the medium. Friend and Hastings (9), in describing the use of neutralized serum, appear to have effected complete removal of bicarbonate before employing this medium, but the same cannot be said of the experiments of Canzanelli *et al* (3). With guinea pig liver, these authors report very large stimulating effects (average value 126 per cent) for both neutralized serum and its ultrafiltrate, and it appears likely that some of this effect was due to unneutralized bicarbonate in the medium. Even so, the stimulating effect is considerably larger than the average figure of 58 per cent which we have obtained for the ultrafiltrate- $\text{CO}_2$  effect with similar material. This discrepancy might be accounted for by their finding a lower absolute value for liver  $Q_{\text{O}_2}$  in Ringer-phosphate medium. In point of fact, however, their absolute values are rather higher than ours, so that the lack of quantitative agreement must be attributed to genetic differences in the guinea pigs or to nutritional factors which, as they found, might play a rôle.

The incomplete removal of bicarbonate from neutralized serum is also responsible for our earlier report (22) that the  $Q_{\text{O}_2}$  of rabbit bone marrow is the same in neutralized serum as in serum- $\text{CO}_2$ . The data of Table III now disclose that in  $\text{CO}_2$ -free serum, the rate of respiration averages about one-third less than in serum- $\text{CO}_2$ . It is perhaps worthy of note, however, that if simple Warburg manometers must be employed, the full stimulating effect of serum may still be obtained, at least with this tissue, by using partially neutralized serum.

Finally, mention may be made of a possible bearing of this work on the shock problem. Several groups of investigators in this field (15, 16) have

commented on the existence, both in normal plasma and in that obtained from animals subjected to burns, of substances that increase tissue respiration. Although it is unlikely that changes in plasma bicarbonate in shock are of sufficient magnitude to influence tissue metabolism (except indirectly by pH changes), an awareness of the specific effect of bicarbonate on respiration is essential for proper management of tissue metabolism experiments in this field. Obviously, particular attention should be directed to identification of the substances responsible for the effect of  $\text{CO}_2$ -free ultrafiltrate. The present investigation is being extended along these lines.

#### SUMMARY

1 In confirmation and extension of the results of other workers, it has been found that the rate of respiration of various mammalian tissues is of the order of 20 to 40 per cent higher in Ringer-bicarbonate- $\text{CO}_2$  medium than in Ringer-phosphate medium. The magnitude of this bicarbonate effect, however, varies over a wide range in individual experiments. Rabbit kidney cortex is exceptional among the tissues studied in exhibiting a slightly lower, rather than higher rate of respiration in the bicarbonate  $\text{CO}_2$  medium.

2 The "stimulating" effect of bicarbonate may be observed in simple Warburg manometry in which respiratory  $\text{CO}_2$  is absorbed by alkali. Under these conditions, the bicarbonate content of the medium falls to low levels during the experiment and may even reach zero without the augmented rate of respiration being affected. This suggests the formation within the tissues of a stable substance which maintains respiration at an elevated rate.

3 Only small amounts of bicarbonate (of the order of 3 mm per liter) need to be present at the beginning of the experiment in order to exert a maximal effect. Consequently, in employing neutralized serum in tissue respiration studies, special precautions must be taken to remove all the bicarbonate, if its effect is to be eliminated.

4 In addition to bicarbonate, serum and its ultrafiltrate contain other metabolically active substances that augment tissue respiration. The identity of these substances remains to be elucidated. Their effect summates more or less completely with the effect of bicarbonate, depending upon the tissue employed and whether the suspension medium is serum or its ultrafiltrate.

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# CYSTINE DETERMINATION IN PROTEINS AND FOODS\*

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Among the cysteine reactions, Sullivan's (1) is the most specific. In the past, numerous workers (2-5) have found that the method based upon this reaction, when applied to pure cystine solutions, has failed to produce color intensities proportional to the quantities of cystine tested. Although attempts have been made to modify the method (6, 7) in order to correct this difficulty, none of the suggested modifications produced true proportionality as claimed. While most of these efforts have been confined to methods involving comparison with a visual colorimeter, photometric methods have also been tried.

Krijgsman and Bouman (2) and also Bushill, Lampitt, and Baker (3), in 1934, used the Pulfrich photometer in the Sullivan method. Under the conditions of their experiments, the characteristic red "color complex" for cysteine was always contaminated with extraneous colored material originating from excess sodium  $\beta$ -naphthoquinone sulfonate<sup>1</sup>. Consequently, their absorption spectra and "extinction coefficients" were not those for the red color complex only, but rather for mixtures of the red color complex and a yellow substance derived from excess naphthoquinone. Consonant with the inaccurate extinction coefficient, their choice of 570 m $\mu$  and 530 m $\mu$ , respectively, does not represent the wave-length of minimum transmission of the red color complex.

## EXPERIMENTAL AND DISCUSSION

Our difficulty in obtaining proportionality in colors by the use of the Duboscq colorimeter in Sullivan's method prompted us to use the spectrophotometer. We determined the most suitable wave-length for measuring the intensity of the red color complex by preparing transmission spectra of solutions that contained no extraneous colored material. Because there is no description in the literature of a method for the isolation and purification of the red color complex, we employed a procedure which served the

\* This is the seventh in a series of papers on amino acids in staple foods.

<sup>1</sup> For convenience hereafter we will use the term "naphthoquinone" for this reagent.



Although we succeeded in obtaining a color intensity proportional to the pure cystine by our spectrophotometric technique of Sullivan's method, we could not establish proportionality when this technique was applied to a protein hydrolysate (Table II, Series A). In the hydrolysates the calculated quantities of cystine decreased with increase in the size of aliquot taken for analysis. By separating the cystine in the form of cuprous mercaptide from the hydrolysate, a procedure introduced by Rossouw and Wilken-Jorden (5) in connection with the Sullivan method, and treat-

TABLE II

*The Effect of the Presence (Series A) and Absence (Series B) of Non Cystine Constituents of Protein Hydrolysate on Cystine Values*

Series	Experiment No	Material	Hydrolysate used*	Cystine
			cc	per cent
A	1	50 mg zein	5	0.80
	2	100 " "	10	0.72
	3	30 mg egg albumin	1	1.1
	4	78 " " "	2.6	0.57
	5	Whole egg	4	121†
	6	" "	8	97†
B	7	50 mg zein	5	0.72
	8	76 " "	7.6	0.71
	9	90 " egg albumin	3	0.87
	10	180 " " "	6	0.81
	11	180 " soy bean protein (globulins)	3	1.60
	12	360 " " " "	6	1.50
	13	Whole egg	2	133†
	14	" "	2.6	134†
	15	" "	4.0	132†

\* Hydrolysate volumes were brought to 5 cc in every case either by evaporation or by addition of water.

† The figures represent quantities of cystine in mg. calculated for the whole egg.

ing it as we did pure cystine, we obtained substantially the same result regardless of the size of the aliquot as shown in Series B of Table II.

The procedure for the determination of cystine in proteins and in food materials is as follows:

The protein material is hydrolyzed in 20 per cent HCl for 24 hours, the solution filtered, and the humin washed with hot dilute HCl. The combined hydrolysate and washings containing 4 or 5 mg of cystine, or an aliquot containing that quantity, are transferred to a long Pyrex test tube containing one or two glass beads and excess HCl is removed by evaporating over a direct flame to a syrupy consistency. This procedure is re-

peated twice after the addition of 5 cc of water each time and the residue made up to a volume of 25 cc (Solution A)

To a 50 cc centrifuge tube are transferred 2.5 cc of glacial acetic acid, 3.5 cc of 5 *N* NaOH, and a quantity of distilled water sufficient to bring the final volume to 40 cc after the test Solution A has been added. The mixture is cooled to room temperature and an aliquot of Solution A containing 2 to 4 mg of cystine is added. The mixture at this stage has a pH value of approximately 4.2.

10 drops of a cuprous chloride solution, prepared according to the directions of Rossouw and Wilken-Jorden (5), are added dropwise with constant stirring. After 40 minutes the precipitate is separated by centrifugation, the supernatant liquid discarded, and the precipitate suspended in 20 cc of absolute alcohol. The suspension is stirred frequently during the following 10 minutes, after which the precipitate is again separated by centrifugation and the supernatant solution is discarded. The precipitate is dissolved in 1 per cent HCl, with 5 cc in three portions, and the solution transferred into a 10 cc volumetric flask. 0.5 cc of pyridine is added, the solution mixed, and then 1 cc of 10 per cent KCNS is added to precipitate the copper. The centrifuge tube is washed with several portions of distilled water to bring the volume to 10 cc (Solution B).

5 cc of Solution B are transferred into a 16 cc volumetric flask. To this solution are added 2 cc of a 5 per cent NaCN solution in 0.5 *N* NaOH and 10 minutes after 1 cc of an aqueous solution containing 3 mg of sodium  $\beta$ -naphthoquinone-4-sulfonate is added, followed in 10 seconds by 5 cc of a 10 per cent solution of Na<sub>2</sub>SO<sub>3</sub> in 0.5 *N* NaOH. The color is permitted to develop for 25 minutes, after which 2 cc of 5 *N* NaOH and 1 cc of a 2 per cent solution of sodium hydrosulfite in 0.5 *N* NaOH are added. The volume is adjusted to 16 cc by addition of a few drops of distilled water if necessary, and the solution is thoroughly mixed. Reading in the spectrophotometer should be made within 2 minutes at the wave-length 505  $m\mu$ .

While the color development proceeds, the necessary adjustments on the spectrophotometer are made. We used a Coleman spectrophotometer, model 10-S, adjusted to have a monochromator slit of 7.5  $m\mu$  in width. The spectrophotometer is calibrated against a blank of distilled water, since at 505  $m\mu$  water and a blank containing less than 3 mg of naphthoquinone give 100 per cent transmission. Aliquots of Solution B containing 0.8 to 3.2 mg of cystine were read in a 13.2 mm square cuvette containing an 11 mm square prism. With more dilute solutions the prisms were omitted. In time the color intensity of the red complex decreased. For this reason, readings were made within 1 or 2 minutes after the addition of hydrosulfite.

A calibration curve (log per cent  $T$  against concentration) is prepared by treating a series of solutions of pure cystine in the same manner as Solution B. The cystine values of the test solutions are calculated from this curve and expressed in percentages calculated on a moisture and ash free basis.

#### SUMMARY

1 It was found that the wave-length of minimum transmission for the red "color complex" produced in Sullivan's method for cystine is 505 m $\mu$ .

2 Evidence is presented of the stoichiometric relationship between naphthoquinone and cystine in Sullivan's cysteine reaction.

3 It was shown that the color produced in Sullivan's cysteine reaction follows Beer's law.

4 A spectrophotometric method for the determination of cystine in proteins and food materials is described, representing a modification of the Rossouw and Wilken-Jorden mercaptide precipitation and the Sullivan colorimetric method.

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# PHENOL CONJUGATION

## II THE CONJUGATION BY RAT AND CAT TISSUES *IN VITRO*\*

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Interest in the origin and fate of phenol in the animal body has arisen since Staedeler (1) discovered volatile phenols in the urine of horse, cow, and man. Although the types of conjugation are known, the mechanisms involved are still obscure.

Baumann (2) and Christians and Baumann (3) were the first to deal with the problem of the site of phenol conjugation in the animal body. Later, using the method of organ perfusion, Embden and Glaessner (4) arrived at the conclusion that phenol conjugation occurred in dog liver, to a small extent in kidney and lung, and not at all in muscle. Many workers have dealt with this problem, recently, for instance, Marenzi (5), Barac (6), Hemingway, Pryde, and Williams (7), Bernheim and Bernheim (8), Lipschitz and Bueding (9), and ourselves (10).

In a previous paper (10) we reported some studies concerning phenol conjugation by rat tissues *in vitro*. With the purpose of arriving at a better understanding of the process, we have studied the conjugation of phenol under different conditions and in the presence of certain inhibitors.

### Methods

The animal (rat or cat) was killed by a blow on the head and the tissues were immediately taken out and sliced according to the Warburg technique. The tissue slices were incubated for 2 hours (unless otherwise stated) at 37.5° in Krebs' (11) solution, pH 7.2, phosphate buffer, containing 0.25 or 0.5 mg of phenol in 100 ml, and 0.2 gm of glucose in 100 ml, in 50 ml Erlenmeyer flasks containing 15 ml of solution. The gas phase contained 100 per cent oxygen and the flasks were shaken 100 times a minute. The weight of tissue per flask is stated in the tables.

Total and free phenols were determined according to the Theis and Benedict method as applied to the Pulfrich photometer by Marenzi (12). Some

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of the later determinations were performed with the same Theis and Benedict technique, adapted for use with the Evelyn photocolormeter, except for Filter 490, but gum acacia was not added. It was found necessary to neutralize the excess trichloroacetic acid of the filtrates in order to obtain accurate results. All phenol values were calculated as micrograms in 15 ml of solution.

*Conjugation by Different Rat Tissues*—Phenol conjugation in liver, kidney, intestine, and diaphragm has already been described (10).

TABLE I

*Phenol Conjugation by Animal Tissues in Vitro*

Medium, Krebs' solution, pH = 7.2, phosphate buffer, glucose 0.2 gm per 100 ml, phenol 0.5 mg in 100 ml. Gas phase, oxygen, incubation time, 2 hours. Volume of solution in each flask, 15 ml.

Animal	Tissue	Conjugation coefficient*	Dry weight of tissue
			mg
Rat	Adrenal	0 (2)	5 -20
	Brain	0 (10)	10 -14
	Heart	0 (9)	3.6 - 5.4
	Ovary	0 (3)	10 -15
	Spleen	$3.5 \pm 0.2$ (6)	2.2 - 3
	Stomach	0 (11)	22 -40
	Testicle	0 (10)	8 -18
	Uterus	0 (2)	20 -38
Cat	Liver	6	4
	"	2.6	5.2
	Kidney	4	6
	"	1.9	5.4
	Small intestine	0.2	110
	" "	0.23	164

\* Micrograms of phenol conjugated per mg of dry weight of tissue in 2 hours. The figures in parentheses indicate the number of determinations.

The results obtained with adrenals, brain, heart, ovary, spleen, stomach, testicle, and uterus are given in Table I. Spleen is the only tissue, besides liver and intestine, that conjugated phenol. No tissue has been found to contain conjugated phenols if incubated without phenol.

*Conjugation by Cat Tissues*—The only tissues studied were liver, kidney, and small intestine. Table I shows the results. As in the rat, liver and small intestine conjugated phenol. Kidney which does not conjugate phenol in normal rats (as determined by our technique) appeared to conjugate as much as liver in the two cats studied.

*Conjugation by Tissue Brei*—Rat liver and large and small intestine brei did not show ability to conjugate phenol. As the synthesis of conjugated

phenols probably requires some other simultaneous reaction that will provide the required energy, it was of interest to determine whether the brei of a tissue that conjugates, and therefore contains the enzyme concerned, and slices of a tissue that does not normally conjugate, but might perform the energy-yielding or coupled reaction, could, if incubated together, cause conjugation. All the attempts made were unsuccessful, there was no conjugation when liver, small intestine, and large intestine brei was used with diaphragm strips or kidney slices.

*Rats Fed Phenol or Borneol*—The fact that the concentration of a certain enzyme increases in bacteria when the corresponding substrate is added to the medium is well known (13). The same has been shown to be true

TABLE II

*Influence of Phenol Administration on Phenol Conjugation by Rat Tissues in Vitro*

Medium, Krebs' solution, pH = 7.2, phosphate buffer, glucose 0.2 gm per 100 ml, phenol 0.5 mg in 100 ml. Gas phase, oxygen, incubation time, 2 hours. Volume in each flask, 15 ml.

Tissue	Conjugation coefficient*	Dry weight of tissue	Conditions
		mg	
Liver	2.19 ± 0.1 (46)	3-10	No phenol
"	5.28 ± 0.48 (5)	5-8	Phenol
Kidney	0 (16)	3-20	No phenol
"	5.94 ± 1.5 (5)	3-7	Phenol
Small intestine	1.18 ± 0.12 (17)	13-55	No phenol
	1.45 ± 0.34 (4)	22-40	Phenol
Large "	1.24 ± 0.13 (17)	12-40	No phenol
	1.55 ± 0.39 (4)	19-34	Phenol

\* Micrograms of phenol conjugated per mg of dry weight of tissue in 2 hours. The figures in parentheses indicate the number of determinations.

concerning enzymes of mammalian tissues, saccharase by Weinland (14), arginase by Lightbody and Kleinman (15), alcohol dehydrogenase by Leloir and Muñoz (16), and  $\beta$ -glucuronidase by Fishman (17). We therefore fed borneol and phenol to rats to see whether the ability to conjugate phenol would increase.

The rats weighed from 130 to 290 gm and were fed a phenol solution (0.6 to 0.7 gm of phenol in 100 ml) by stomach tube every 2 or 3 days, four to sixteen times. The total amount of phenol fed every time has been from 0.01 to 0.06 gm per kilo of body weight. In Table II are shown the results on different tissues compared with the value for conjugation in tissues of rats that had not received phenol. Rats were kept with the usual diet of bread and milk.

From Table II it is clear that the ability to conjugate phenol increases in liver and kidney after phenol feeding. Lipschitz and Bueding (9) have shown that normal rat kidney conjugates phenol to a slight extent. This was not detectable by our method. Because of the few determinations made and the irregularity of results, no conclusion is possible concerning the intestine.

Four other rats were fed borneol dissolved in olive oil (0.025 gm per ml). They received four to seven doses of 0.25 gm per kilo of body weight.

The conjugation coefficient for liver was found to be  $3.75 \pm 0.18$  (twenty-three determinations) and for kidney  $3.24 \pm 0.29$  (five determinations). Comparison of these results with the values obtained with rats that did

TABLE III

*Influence of Sulfate on Phenol Conjugation by Rat Tissues in Vitro*

Medium, Krebs' solution, pH = 7.2, phosphate buffer, glucose 0.2 gm per 100 ml, phenol 0.5 mg in 100 ml. Gas phase, oxygen, incubation time, 2 hours. Volume in each flask, 15 ml.

Tissue	Conjugation coefficient	Dry weight of tissue mg	Condition	Strain
Liver	$1.62 \pm 0.21$ (11)	3-8	No sulfate	Rosario Medical School
"	$2.19 \pm 0.1$ (46)	3-10	With sulfate	
Small intestine	$1.3 \pm 0.4$ (2)	13-30	No sulfate	
Large "	$1.18 \pm 0.12$ (17)	13-55	With sulfate	Vanderbilt (Duke)
	1.67 (1)	12	No sulfate	
Liver†	$1.24 \pm 0.13$ (17)	12-40	With sulfate	
	$0.07 \pm 0.02$ (10)	10-21	No sulfate	
"	$0.39 \pm 0.03$ (21)	8-27	With sulfate	

\* Micrograms of phenol conjugated per mg of dry weight of tissue in 2 hours. The figures in parentheses indicate the number of determinations.

† Optimum incubation time 1 hour, conjugation coefficient calculated for 1 hour.

not receive borneol (liver  $2.19 \pm 0.1$  (forty-six determinations) and kidney 0 (sixteen determinations)) leaves no doubt that the ability to conjugate phenol increases in liver and kidney after borneol feeding. The rats used for these experiments conjugated phenol in the absence of sulfate.

*Influence of Sulfur Compounds*—Baumann (2) in 1876 observed that phenol was excreted as an ester of sulfuric acid, and his result has been confirmed several times. We attempted to see if sulfates had some effect on phenol conjugation *in vitro* by substituting magnesium chloride in equivalent concentration for magnesium sulfate in Krebs' solution (11).

With the rats used at the Rosario Medical School there was a slight but noticeable decrease in conjugation (Table III), whereas the rats used at

Duke University (Vanderbilt strain (18)) have shown practically no conjugation in absence of sulfates (Table III). This last result agrees with the findings of Bernheim and Bernheim (8) in the guinea pig.

Another interesting point is the great difference in conjugating power of both strains, as can easily be noticed from Table III.

We tried to use glutathione but it interferes with phenol determination. It is possible that in absence of sulfates, phenol may be conjugated with glucuronic acid *in vitro*.

*Influence of Inhibitors*—Lipschitz and Bueding (9) have shown that the formation of conjugated glucuronic acids is inhibited by monoiodoacetic acid. We found the same effect on phenol conjugation of monoiodoacetic

TABLE IV

*Influence of Monoiodoacetate on Phenol Conjugation by Rat Tissues in Vitro*

Medium, Krebs' solution, pH = 7.2, phosphate buffer, glucose 0.2 gm per 100 ml, phenol 0.25 mg in 100 ml. Gas phase, oxygen, incubation time, 2 hours. Volume of solution in each flask, 15 ml.

Tissue	Total phenols	Free phenols	Conjugated phenols	Dry weight of tissue	Conjugation coefficient*	Condition
	$\gamma$	$\gamma$	$\gamma$	mg		
Liver	38.6	33.5	5.1	11.2	0.46	No MIA†
"	40	40.4	-0.4	11	0	0.002 M MIA
Small intestine	188	143	45	30	1.05	No MIA
	188	188	0	25	0	0.002 M MIA
Large "	188	155	33	25.4	1.3	No MIA
	155	155	0	27.2	0	0.002 M MIA
Liver	49	49	0	15.5	0	MIA + lactate
"	56	43	13	10	1.3	" + glucuronate
Small intestine	187.5	187.5	0	32	0	" + lactate
Large "	129	129	0	32	0	" + "
	154.5	137	17.5	25	0.7	" + glucuronate

\* Micrograms of phenol conjugated per mg of dry weight of tissue in 2 hours.

† MIA = monoiodoacetate.

acid at a concentration of 0.002 M. The addition of lactate (we used recrystallized lithium lactate at a concentration of 106 mg in 100 ml) to the monoiodoacetic acid treated tissue did not restore the conjugating power. On the contrary, glucuronic acid<sup>1</sup> (10 mg in 100 ml) reestablished conjugation in the treated tissue (Table IV). This was also done on the strain of rats which are able to conjugate in the absence of sulfate. Ascorbic acid<sup>2</sup> had no effect on the conjugation.

<sup>1</sup> Prepared according to Quick's method (19).

<sup>2</sup> Ascorbic acid interferes with phenol determination, but can be destroyed by bubbling O<sub>2</sub> for 15 minutes after the solution is made alkaline with 2 drops of N NaOH per 15 ml.



We have shown (10), as have Bernheim and Bernheim (8), Lipschitz and Bueding (9), and Hemingway, Pryde, and Williams (7), that either 0.001 M cyanide or absence of oxygen ( $N_2$  atmosphere) stops conjugation. The addition of lactate or glucuronic acid did not reestablish conjugation under those conditions.

### Results

It is well known that phenol conjugates in the animal body with sulfuric and glucuronic acids. In both cases, besides the enzyme systems involved in synthesis, there is probably a need for a coupled reaction that will provide the energy for the formation of the conjugate. In the case of glucuronic acid conjugation, glucuronic acid must be formed, and according to Quick (20) and Lipschitz and Bueding (9) it is synthesized from products of carbohydrate breakdown (probably trioses).

The absence of conjugation in anaerobiosis and cyanide inhibition indicates the need of an oxidative coupled reaction for the process, as sulfate ion is available and the enzyme is present. The inability of  $\beta$  glucuronidase *in vitro* to synthesize significant amounts of borneolglucuronic acid (Houet, Duchateau, and Florkin (21), Florkin, Crismer, Duchateau-Bosson, and Houet (22)) might be the result of the absence of the coupled reaction which provides the energy. The cell structure is undoubtedly important, as tissue brei does not conjugate phenol.

The inhibition of conjugation by monoiodoacetic acid seems to be due to the inhibition of glucuronic acid formation, as this compound is able to reestablish conjugation after monoiodoacetic acid inhibition. Our results suggest the possibility of glucuronic acid combining directly with phenol to form the conjugate.

The decrease or disappearance of conjugating power, in the absence of sulfates, in one of the two strains of rats used, indicates that they conjugate phenol mainly to sulfate, unless the presence of  $SO_4^{2-}$  ion is necessary for glucuronic acid conjugation, which is doubtful.

### SUMMARY

1. Of the rat tissues studied, liver, intestine, and spleen conjugate phenol, adrenals, brain, diaphragm, heart, kidney, ovary, stomach, uterus, and testicle do not.

2. In the cat, phenol is conjugated by liver, kidney, and small intestine, the only tissues studied.

3. Tissue brei does not conjugate phenol, even in the presence of slices of non-conjugating tissues.

4. After phenol and borneol are fed to rats, phenol conjugation increases in liver and in kidney.

5 In one strain of rats studied, phenol conjugation almost disappears in the absence of sulfates

6 0.002 M moniodoacetic acid inhibits phenol conjugation, which is reestablished by addition of glucuronate, but not of lactate

7 Glucuronic acid does not reestablish conjugation after cyanide inhibition or under anaerobic conditions

8 Ascorbic acid has no effect on the conjugation

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# SEDIMENTATION VELOCITY AND ELECTRON MICROGRAPHIC STUDIES OF INFLUENZA VIRUSES A (PR8 STRAIN) AND B (LEE STRAIN) AND THE SWINE INFLUENZA VIRUS\*

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The results of studies on the purification of the influenza virus, two human types, influenza viruses A (PR8 strain) and B (Lee strain), and the swine type, have been described in recent reports (1-3). From sedimentation data obtained with the analytical ultracentrifuge and from electron micrographs, the three types of virus were found to be ovoid or rounded particles of about 80, 100, and 80  $\mu$ , respectively. Chemical analyses by Taylor (4) showed that the three types of virus are of essentially similar constitution, consisting approximately of lipid 24 per cent, protein 65 per cent, carbohydrate 10 per cent, and nucleic acid of the desoxypentose type 2 to 4 per cent.

Contrary to the findings with several other purified animal (5, 6) and plant (7) viruses, the influenza viruses are not monodisperse. In any given preparation, the particles vary not only in size but in shape as well, as seen in electron micrographs. Despite these variations, however, the electron micrographs showed that the particles of a given type of the virus were of the same sort, and the mean image size in electron micrographs and the mean sedimentation rate in the ultracentrifuge remain constant for all preparations of any one type thus far studied. Differences have been observed in sedimentation constants with the different types of virus, and a dependence of sedimentation rate on virus concentration has been noted.

In the present work, further studies were made of the three types of influenza virus with the analytical ultracentrifuge and the electron micro-

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scope. Analyses were made of the distribution of particle sizes and the mean particle sizes determined by the two methods, and the findings were compared directly.<sup>1</sup> Studies were made also of the variation in the rate of sedimentation with virus concentration.

### *Materials and Methods*

The viruses were egg-adapted strains of influenza viruses A (PR8 strain) and B (Lee strain) obtained from Dr. Thomas Francis, Jr., and the swine influenza virus obtained from Dr. John F. Enders. The purified viruses used for all of the work were obtained from virus-infected chorioallantoic fluid of chick embryos. Purification in most instances was effected by adsorption of the virus on and elution from chicken red blood cells, combined with ultracentrifugation, as previously described (1-4). Other methods used for obtaining virus for special studies will be indicated separately in connection with the particular experiments.

For electron microscopy, an RCA type B electron microscope was used, and all materials were photographed by transmission of electrons through virus preparations made on collodion films in the usual manner. 55 kilovolt electrons were used, and pictures 2 inches square were made, in which the magnification was usually about  $7800\times$ . Further magnification was accomplished by photographic enlargement. Calibration of the electron microscope for previously reported work from this laboratory was made by comparing the images of the virus particles in question with images of the rods of tobacco mosaic virus, whose width has been calculated from x-ray data (8, 9) to be  $15\text{ m}\mu$ . Since the resolving power of the instrument is about  $5\text{ m}\mu$ , the accuracy of image measurements on the width of tobacco mosaic virus rods is limited by some percentage error which is proportional to the ratio  $5/15$ . In comparisons of groups of different particles approximately  $15\text{ m}\mu$  in diameter, the relative sizes may be obtained accurately, since about the same percentage error exists in each case. When measurements are made on larger viruses such as those of influenza, however, the percentage error will be much smaller, i.e. proportional to  $5/100$  or  $5/80$ , and calibration with a small object leads to error. For this reason, a separate calibration was made for measurements on the sizes of the influenza viruses. An object screen of the standard type, but with small holes (about  $76\text{ }\mu$  in diameter), was coated with collodion in the usual manner, and dust particles of irregular but sharp outline were observed in an area outlined by the screen surrounding one of the holes. This area, including the hole in the metal screen, was photographed at a magnification of about  $700\times$  with electrons. The screen was removed from the electron micro-

<sup>1</sup> A part of this work was reported briefly before the meeting of the American Society of Electron Microscopists at New York, January 13, 1944.

scope, and the diameter of the same hole was carefully measured with a calibrated light microscope. This established the magnification as  $689\times$  for this particular setting of the electron microscope. The screen was returned to the electron microscope, and the dust particles framed by the hole in the screen were then photographed at other magnifications up to  $3270\times$ , at which point it was necessary to change to the longer object holder used to photograph the influenza virus. With this in place, the process was continued throughout the range of the instrument. Included in the probable error of this absolute calibration at  $7800\times$  was the error in measurement of the  $76\mu$  hole with the light microscope, an error which should be small, the error in the measurements on the images of the dust particles on two-step calibration plates, which should also be small, and the error introduced by image distortion in the electron microscope itself when the same object was photographed at different magnifications. The last is somewhat greater than the other errors. The absolute sizes calculated for the influenza virus particles based on this calibration should represent more nearly the true values than those calculated from comparisons with the small tobacco mosaic virus rods.

Purified virus concentrates were diluted to the proper concentration for preparation of the collodion mounts for electron micrography by using Ringer's solution in which the virus was purified, or in some cases by using  $0.023\text{ M CaCl}_2$  solution. The latter diluent increases the contrast in electron micrographs of some viruses (6), and improvement in this quality was seen for the influenza virus (1-3) also.

Sedimentation velocity studies in the present work were made in an air-driven ultracentrifuge, whose rotor carried a cell 4 mm thick and 12 mm high at a mean radius of 6.5 cm. The analyses for the determination of sedimentation constant and the distribution of particle size were made from refractive index recordings by the scale method of Lamm. This procedure proved preferable to the absorption method used in previous work (1-3) after it was found that sufficient virus concentration could be employed to permit satisfactory scale line deflections without excessive absorption and scattering of the light.

Particle size distributions have been calculated from the curves of the refractive index gradient of the sedimentation velocity diagrams by the method outlined by Kraemer (10). It has been assumed that the refractive index of the virus solution varies in a linear fashion with virus concentration and that it is independent of particle size. Although it is recognized that the latter assumption may introduce some error, it is probably not greater than that encountered in analogous assumptions which would be necessary if the light absorption method were used for photography of the sedimenting boundary.

*Experiments and Results*

Representative Lamm diagrams of the three types of influenza viruses, A and B and the swine virus, are shown in Figs 1 to 3 respectively. In all cases the rotor speed was  $166\frac{2}{3}$  R P S and the time interval between scale photographs was 5 minutes. Rotor temperature was room temperature, which varied over the range of  $21-29^{\circ}$  for the various experiments and for which suitable corrections have been made in the calculation of the sedimentation constants. All calculations of sedimentation constants were made by using the distances on the abscissa measured to the maximum ordinate of the boundary curves.

The Lamm diagrams showed that the three types of virus sedimented with a single, slightly diffuse boundary, as was observed in the absorption

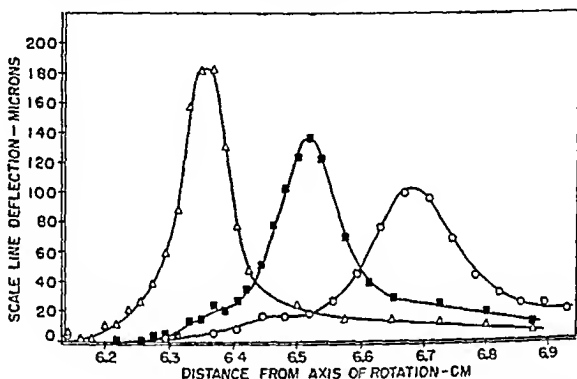


FIG 1 Influenza virus A (PR8 strain) The boundary, sedimenting in a centrifugal field of 7270  $g$ , is shown in three positions by three scale curves taken at intervals of 5 minutes. The virus concentration was 2.43 mg per ml, and the scale distance was 10 cm.

diagrams previously reported (1-3). This character of diffuseness of sedimenting boundary has been seen in all of more than thirty preparations which have been analyzed irrespective of the mode of preparation. Sedimentation constants calculated from a series of diagrams like those of Figs 1 to 3 revealed a physical difference in the three types. When the influence of concentration on rate of sedimentation was disregarded, it was found that the swine virus sedimented (20 mg per ml) about 5 per cent more slowly than did the influenza virus A and about 19 per cent more slowly than influenza virus B at the same concentration. These values are essentially similar to those obtained (1-3) by the absorption method of Svedberg.

The sedimentation rates were, however, not independent of virus concentration in the centrifuge cell. In Fig 4 are shown graphically the variations

in the sedimentation constants calculated from measurements on the sedimentation velocity diagrams of the three types of the virus taken at concen-

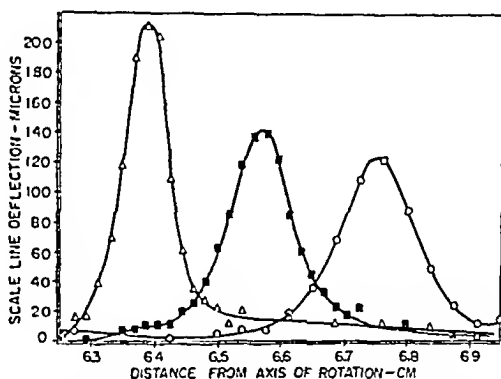


Fig 2 Influenza virus B (Lee strain) The boundary, sedimenting in a centrifugal field of 7270  $g$ , is shown in three positions by three scale curves taken at intervals of 5 minutes The virus concentration was 1.75 mg per ml, and the scale distance was 10 cm

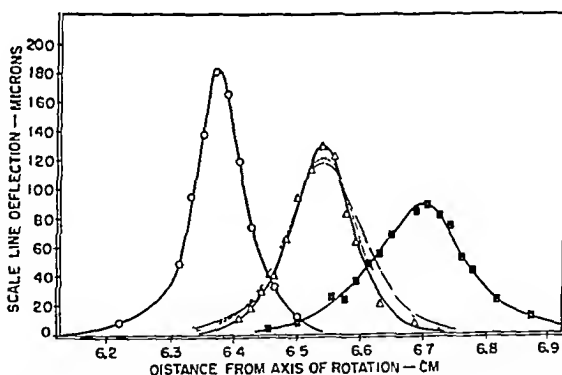


Fig 3 Swine influenza virus The boundary, sedimenting in a centrifugal field of 7270  $g$ , is shown in three positions by three scale curves taken at intervals of 5 minutes The virus concentration was 3.21 mg per ml, and the scale distance was 10 cm In the center two additional curves are shown These were obtained by calculation from the last (dotted line) and the first (dot and dash line) curves of the diagram Their similarity to the observed (continuous line) curve is an indication that diffusion was negligible in its contribution to progressive boundary spread

trations varying from 0.5 to 3.0 mg per ml These limits of concentration were imposed by the Tyndall effect at high concentrations and lack of



sufficient scale line deflection at the low In this region the sedimentation rates of the influenza virus A and the swine virus were linear with concentration, increasing with dilution. The influenza virus B behaved similarly when the virus was in Ringer's solution, but when it was dispersed in Ringer- $\text{CaCl}_2$  solution (1) the influence of concentration was negligible except at the highest concentration level. The reason for this is not now clear, as Ringer- $\text{CaCl}_2$  solution had no such effect on the swine virus or on influenza virus A.

A common feature of Figs 1 to 3 is the spreading of the boundary as sedimentation progresses, as previously noted in absorption diagrams of influenza virus A (PR8 strain) (1), influenza virus B (Lee strain) (2), and

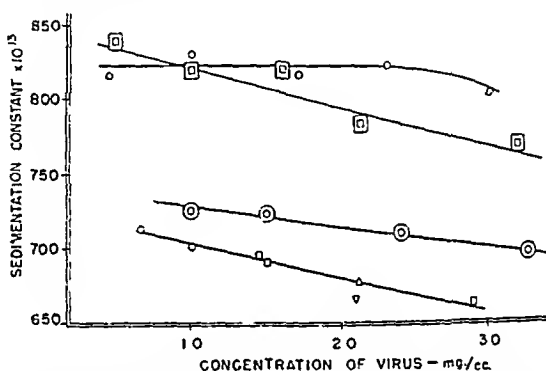


FIG 4. Relation of sedimentation constants of influenza viruses A and B and the swine influenza virus to virus concentration. Influenza virus B (double squares), five dilutions of a single virus preparation in Ringer's solution; (circles), five dilutions of a single virus preparation in Ringer- $\text{CaCl}_2$  solution (1). Influenza virus A (double circles), four dilutions of a single virus preparation in Ringer's solution. Swine influenza virus (circles, squares, and triangles), measurements on three different preparations at various concentrations of virus in Ringer- $\text{CaCl}_2$  solution (1).

the swine influenza virus (3). In order to determine the extent to which diffusion might contribute to this progressive boundary spread, the first and third curves of Fig 3 were transferred to the mean position by calculations described by Signer and Gross (11). The method takes no account of diffusion, assuming uniform sedimentation in a radial force field in a wedge-shaped cell whose sides are radii of the rotor. The similarity of the calculated curves of Fig 3 to the one observed removes all doubt of appreciable diffusion taking place in the short time involved.

Progressive spreading of the sedimenting boundary with negligible diffusion must be due to dissimilarity among the particles of the virus either in size, density, or shape, or a combination of these. Electron micrographs

previously published (1-3) show each type of the virus to consist of particles having a similar appearance, that is, when several hundred of a given preparation were examined in a single micrograph, they showed similar variations in shape and size. Variations in shape are of little consequence in their effect on sedimentation rate, unless there is a considerable departure from the compact sphere. The micrographs showed no such departure, all particles were either round or slightly bean-shaped. The variation in the size of the various particles of a given preparation was great and was in all likelihood sufficient to account for a large part of the diffuseness of the sedimenting boundary. No information is available relative to possible variation in the density of individual particles.

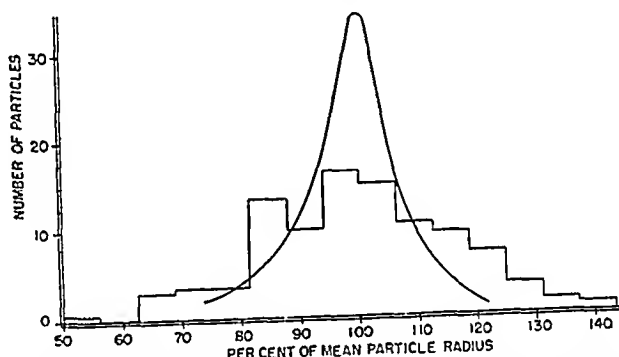


FIG 5 Particle size distribution of influenza virus A. The smooth curve was calculated from sedimentation-velocity data such as those of Fig 1. The step diagram shows the particle size distribution determined from measurements on 107 images in electron micrographs.

The refractive index diagrams of Figs 1 to 3 provide a quantitative estimate of the degree of variation in the sedimentation characteristics of the material comprising the preparations of the respective types of virus. It was of interest to compare these findings with the ultracentrifuge with the results of estimates of particle size distribution obtained from electron micrographs of the virus preparations. In electron micrographs of the sort shown in the previous reports (1-3), measurements were made of image size, and the values obtained are tabulated in the histograms of Figs 5, 6, and 7. In order to do this, the glass negatives were clamped to the stage of a micro-comparator reading in microns, and the image diameters were measured as they came under the cross-hair of the microscope without regard for orientation. The histogram of Fig 5 was constructed from the measurements on 100 images in electron micrographs of influenza virus A.

(PR8 strain) previously reported (1) and on 97 additional images made in the present work. The histogram of Fig 6 includes the data previously

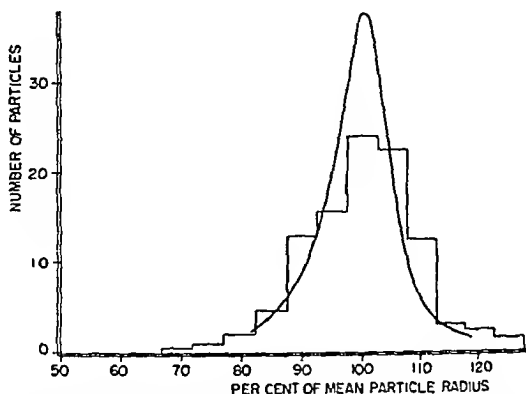


FIG 6 Particle size distribution of influenza virus B. The smooth curve was calculated from sedimentation-velocity data such as those of Fig 2. The step diagram shows the particle size distribution determined from measurements on 198 images in electron micrographs.

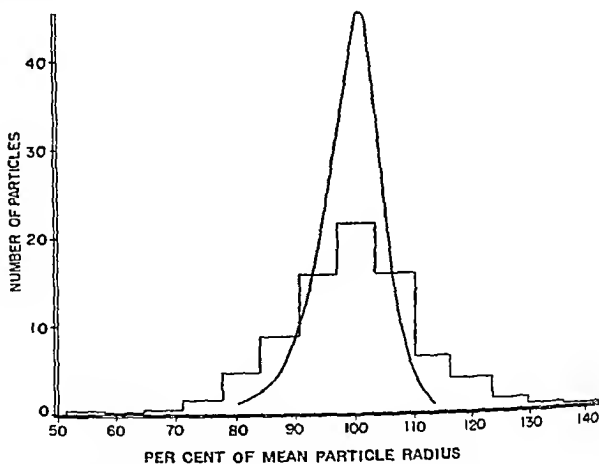


FIG 7 Particle size distribution of the swine influenza virus. The smooth curve was calculated from the sedimentation-velocity curves of Fig 3. The step diagram shows the particle size distribution determined from measurements of 574 images in a single electron micrograph.

described (2) on influenza virus B (Lee strain) and data obtained on 98 images in further study in the present work. In Fig 7, the histogram

relative to the swine virus was based on measurements on 574 images and is the same as that which has already been described (3)

Determinations were then made of the distribution of particle size from the data obtained with the analytical ultracentrifuge. Points determining smooth curves representing the distribution were calculated from the sedimentation velocity curves of Figs 1, 2, and 3 by the method described by Kraemer (10). The curves were then superimposed on the step histograms representing particle size distributions determined with the electron microscope, giving the results shown in Figs 5, 6, and 7. For a direct comparison of these data it was necessary to separate the problem of absolute size measurement in the electron micrographs, which depends on calibration of the instrument, from that of determination of size distribution. This was done by reducing both ultracentrifugal and electron micrographic data to the same coordinates. The abscissae are in terms of per cent of mean particle radius, and the ordinates are proportional to the numbers of particles in any size group. The proportionality factors are arbitrary and determined by making the area under both graphs the same.

The data obtained by the two methods showed marked similarities in the degree of spread for a given type of the virus. In every instance the spread revealed by the measurements of images in the electron micrographs was greater than that indicated by the ultracentrifugal data. The differences seen, however, were perhaps not greater than the error involved in measuring somewhat asymmetrical particles without regard for orientation. From the ultracentrifugal data, there appeared to be some small difference between the respective types of the virus relative to the degree of spread. The swine virus appeared to be the most homogeneous with respect to sedimentation characteristics, and influenza virus A least. These differences between the types of the virus indicated by the ultracentrifuge were reflected also in the spread shown by the histograms.

Estimation of mean particle size can be made from both electron microscopic and ultracentrifugal data. The results of size calculations from the electron micrographs are shown in Table I, based on the ratio of image diameter of the influenza virus to the image diameter of tobacco mosaic virus rods, which have been reported to be  $15\text{ m}\mu$ . The data employed for the respective types of the virus were those obtained from the measurements yielding the histograms of Figs 5, 6, and 7. The values in the last line of Table I were derived from the same micrographs but were calculated on the basis of the direct calibration of the electron microscope, as described above. The values obtained on the basis of the direct calibration are higher than those derived from a comparison with tobacco mosaic virus rods and, consequently, are in less good agreement with the sedimentation velocity results.

For the calculations of size from the ultracentrifugal data the sedimentation constants employed (Table I) were obtained by extrapolation of the curves of Fig 4 to infinite dilution. It was necessary to assume that the virus particle is a sphere and to employ a value for the density of the particles. The values of the particle density used in the present calculations (Table I) were the reciprocals of the partial specific volumes of the respective types of the virus determined by pycnometric measurement. In a previous report (1) of estimations of the size of influenza virus A (PR8 strain) from ultracentrifugal data, the value 0.833 was employed as the partial specific volume. This value was the reciprocal of the density of influenza virus A (WS strain), 1.20, determined by Elford and Andrewes.

TABLE I

*Partial Specific Volume, Sedimentation Constant, and Mean Diameter of Influenza Virus A (PR8 Strain), Influenza Virus B (Lee Strain), and Swine Influenza Virus*

	Influenza virus A (PR8 strain)	Influenza virus B (Lee strain)	Swine influenza virus
Partial specific volume	0.824	0.865	0.850
Sedimentation constant*	742 $\times 10^{-13}$	840 $\times 10^{-13}$	727 $\times 10^{-13}$
Diameter, from sedimentation velocity data, $m\mu$	80.1	100.4	86.8
Image ratio of influenza virus to width of tobacco mosaic virus rods	5.33	6.50	5.12
Diameter from electron micro- graphs, calibration with tobacco mosaic virus rods, $m\mu$	80.0	97.6	76.8
Diameter from electron micro- graphs, direct calibration, $m\mu$	101	123	96.5

\* Values obtained by extrapolation of the curves of Fig 4 to infinite dilution of the virus.

from centrifugal studies on the virus in "broth-sugar" solutions. For the calculations in the present work, the partial specific volume of the influenza virus A (PR8 strain), based on dry weight, was determined by measurements in the pycnometer. In eight determinations on three specimens of the purified virus, the individual values obtained were 0.8240, 0.8225, 0.8240, 0.8239, 0.8236, 0.8234, 0.8225, and 0.8241 and the average was 0.824. The virus content of the preparations on which the measurements were made was from 10.52 to 20.80 mg per ml. The values of partial specific volumes employed here (Table I) for calculations of the sizes of influenza virus B (Lee strain) and the swine influenza virus were those previously reported (2, 3). It should be emphasized that these values were obtained on the

basis of dry weight and that the reciprocals of these values can scarcely be considered to represent the true density of the viruses in solution. Resort to usage of such values is made only in the absence of data on density determined directly on the virus in solution. The mean particle diameters of the respective types of the virus calculated on the basis of the assumptions described above are given in Table I. The respective values differ slightly from those previously reported owing to the use of the sedimentation constants obtained by extrapolation of the lines of Fig 4 and, in the instance of influenza virus A, to the value of the partial specific volume which differed from that previously employed (1).

The results described above were obtained with virus purified by adsorption on and elution from chicken red blood cells, combined subsequently

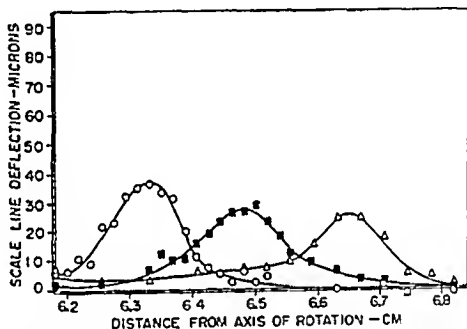


FIG 8 Influenza virus A (PR8 strain) from chorioallantoic fluid concentrated by adsorption on and elution from chicken red blood cells. No differential ultracentrifugation was used for this preparation. The boundary sedimenting in a centrifugal field of 7270  $g$  is shown in three positions by three scale curves taken at intervals of 5 minutes. The scale distance was 10 cm.

with sedimentation and further concentration in the ultracentrifuge. The influenza virus can be concentrated, also, by several methods not involving ultracentrifugation. It was of interest to learn whether the particle size distribution and the mean particle size of the purified virus were substantially different for different methods of purification and concentration. Accordingly, sedimentation velocity studies were made on virus purified by adsorption on and elution from chicken red blood cells and by precipitation with calcium phosphate.

In Fig 8 is shown the scale diagram of influenza virus A obtained by adsorption and elution with red blood cells. The procedure employed for concentration was the same as that previously described (1, 4), except that the volume of fluid used for elution of the virus from the red blood cells

error in the estimation of absolute size, however, would not affect the relative differences seen between the three types

Aside from strain differences in mean particle diameter, there was found for all three viruses a fairly wide distribution of particle sizes about the mean. This distribution was about the same for all three, and it has been measured both with the electron microscope and the ultracentrifuge with essentially similar results. The variations in size ranged roughly 50 per cent above and 50 per cent below the mean, the distribution being approximately normal. The degree of variation apparent from the electron micrographs was somewhat greater than that determined from the ultracentrifugal data. The difference probably was related to the measurement of the images irrespective of orientation on the electron micrographic plates.

It has been suggested (13) that particle size and distribution of particle size of virus purified by centrifugation may be related to differential selection of particles by spinning at alternate low and high speeds. Sedimentation diagrams, Figs 1, 2, and 3, were made from viruses which had been sedimented once in the ultracentrifuge and spun once again at low speed to discard large aggregates. It was apparent that this procedure exerted some selective action, but if the viruses were not homogeneous enough to form these boundaries prior to this limited treatment, they would not be affected, for the difference between high speed and low speed treatment was far too great to be so selective. The low order of selectivity of the centrifugal process is illustrated by the sedimentation velocity diagrams of virus preparations concentrated by the hemagglutination and  $\text{Ca}_3(\text{PO}_4)_2$  methods when ultracentrifugal treatment was not used. The sedimentation diagrams of Figs 8 and 9 show close similarity to those of Figs 1, 2, and 3. Subsequent ultracentrifugal concentration and low speed clarification of the material of Fig 9 did not change its essential character.

An outstanding character of the influenza viruses is their departure from monodispersity, as clearly shown both by the sedimentation velocity and the electron micrographic data. In this respect the influenza viruses are somewhat similar to the elementary bodies of vaccinia (14, 15), but differ from other animal and plant viruses obtained in purified preparations in concentrations great enough to permit adequate studies. Information gained of the degree of heterogeneity of particle size and shape is of considerably greater significance than as solely descriptive of the influenza viruses themselves. Until recently the principal method for ready examination of purified viruses for studies on their physical character was associated with the analytical ultracentrifuge. The data obtained in this way have indicated a high degree of monodispersity for certain of the plant (7, 16) and animal viruses (17, 18) thus far purified, and have been employed for

conclusions with respect to the homogeneity and ultimate nature of these seemingly monodisperse agents. Before access to the electron microscope, the diffuse boundaries seen with the influenza viruses would have been judged as indicating that the preparations did not consist of virus alone. The electron microscope not only confirms the departure from monodispersity to a degree similar to that shown by the ultracentrifuge, but shows clearly that the variations are in biologically similar particles, not in particle kind. It is thus evident that a diffuse boundary obtained with the analytical ultracentrifuge cannot be construed in the absence of other evidence as indicative of heterogeneity of particle kind in virus preparations.

Corollary to the data relative to the departure of the influenza viruses from monodispersity is the conclusion that these agents are not molecular in nature. Because of the homogeneity of preparations of purified rabbit papilloma (17) and the equine encephalomyelitis viruses (18), which give single sharp boundaries in sedimentation velocity diagrams, these agents have been considered to be macro molecules, a concept similar fundamentally in this respect to that held for the plant viruses. Electron micrographs of the animal viruses (5, 6, 19) have consistently corroborated the evidence of homogeneity or the lack of it obtained with the ultracentrifuge. In addition, however, there has been plainly demonstrable in all instances evidence of structure within the virus particles. This variation is seen not only within the substance of the individual particles, the internal structure may vary in form and arrangement from particle to particle. This is not compatible with the concept that these viruses are macro molecules, rather they much more closely resemble the form and structure of certain bacteria (in the instance of the influenza viruses, pure cultures of small coccobacilli). The similarity of the influenza viruses to bacteria is seen further in their chemical constitution, since they are composed of protein, fat, and carbohydrate, the latter bound in complex form outside the nucleic acid portion of the virus particle.

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# THE CHEMICAL ESTIMATION OF $\alpha$ -TOCOPHEROL AND TOTAL TOCOPHEROL IN MIXTURES OF THE $\alpha$ , $\beta$ , AND $\gamma$ FORMS\*

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A chemical method for the estimation of  $\alpha$ -tocopherol in the presence of other naturally occurring tocopherols is greatly needed. The original Emmeric-Engel method (1) for the tocopherols was based upon the quantitative reduction of ferric ions by the tocopherols, and the determination of the amount of ferrous ions thus formed by means of the color reaction with  $\alpha, \alpha'$ -bipyridine. Most variations on this principle deal with the removal of interfering substances and the preliminary treatment of the sample.

The availability of synthetic  $\alpha$ -,  $\beta$ -, and  $\gamma$ -tocopherols allows the investigation of the fundamental color reaction. Baxter, Robeson, Taylor, and Lehman (2) published data on the rates of reactions of the tocopherols with the ferric chloride-bipyridine reagent.  $\alpha$ -Tocopherol had the greatest rate of color development.  $\beta$ - and  $\gamma$ -tocopherols developed color at equal rates, but more slowly than did  $\alpha$ -tocopherol, eventually reaching a more intense color level. At 60 seconds, the three tocopherols had developed equal color intensities. Apparently, no work has been published on the effect of temperature upon the comparative reaction rates of the tocopherols. In view of this, a study was made of the effect of temperature on the rates of color development with pure  $\alpha$ -,  $\beta$ -, and  $\gamma$ -tocopherols. From the results obtained, a method became apparent for the determination of  $\alpha$ -tocopherol in the presence of  $\beta$ - and  $\gamma$ -tocopherols. The factors relating to such a method were derived, and the method was applied to mixtures of the pure tocopherols and to several vegetable oils.

## EXPERIMENTAL

*Effect of Concentration of Reagents on Color Development*—The concentration of ferric chloride and  $\alpha, \alpha'$ -bipyridine was found to be of fundamental importance to the rates of color development with pure tocopherols. Increased concentrations of these two reagents resulted in both an increased rate and an increased maximum color intensity, when constant amounts of the three tocopherols were used. It was also found that the relative proportion of the ferric chloride to the bipyridine was important. Up to a certain

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point, the progressively increased proportion of ferric chloride resulted in an increased rate of color development. Further increases of the ferric chloride were followed by fading of the color initially formed.

By use of appropriate concentrations of the ferric chloride and bipyridine, and with glacial acetic acid (3) as the solvent, the time for equivalent color intensity development for the three tocopherols was brought to 15 minutes when the reaction was carried out at 35°. The color reagent used to give this result was made by dissolving 250 mg of  $\alpha, \alpha'$ -bipyridine and 125 mg of  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$  in 1 liter of reagent glacial acetic acid. To insure standardization and accuracy, a stock solution of the ferric chloride in glacial acetic acid was made up, and aliquots of this were used in preparing the color reagent.

For the most part, all studies to be reported involved the solution of the tocopherol or oil under test in Skellysolve B, purified by treatment with sulfuric acid as outlined by Devlin and Mattill (3). 2 ml of the Skellysolve solution were pipetted into an Evelyn colorimeter tube, and placed at the temperature of the run for 5 minutes. 8 ml of the color reagent, also at the temperature of the run, were rapidly delivered into the tube. Time was recorded with a stop-watch. Readings were taken on the Evelyn photoelectric colorimeter (Filter 540) at the time desired  $\pm 2$  seconds.

*Effect of Temperature on Rate of Color Development*—Standard solutions of synthetic  $\alpha$ -,  $\beta$ -, and  $\gamma$ -tocopherols<sup>1</sup> were made up in Skellysolve B to contain approximately 40  $\gamma$  per ml, in each case. The rates of color development with 1 ml of these standards were determined at 35°, 27°, and 19°. The results are shown in Fig 1. At 15 minutes and at 35° the degree of color developed was about the same for the three tocopherols. At the lower temperature, the rates of color production were much slower in all cases. However, in the case of  $\alpha$ -tocopherol, the rate was still sufficiently rapid for maximum color to be formed by the end of 15 minutes;  $\beta$ - and  $\gamma$ -tocopherols showed only about two-thirds as much color by the end of this time period. The results in Fig 1 offered the basis of a method for the estimation of  $\alpha$ -tocopherol in mixtures of the three tocopherols.

Standard curves were established for each of the three tocopherols at 35° and at 15° with time at 15 minutes. The lower temperature was maintained by use of an insulated bath containing a slush of frozen and liquid glacial acetic acid. The melting point of acetic acid is 16.7°, but apparently there was enough water in the product used to lower the melting point to 14.5–15°. This temperature was maintained with high constancy for long periods of time. For determinations at this temperature, the colorimeter tubes containing the 2 ml aliquots of the Skellysolve solutions

<sup>1</sup> The supplies of the synthetic tocopherols were provided by Merck and Company, Inc.

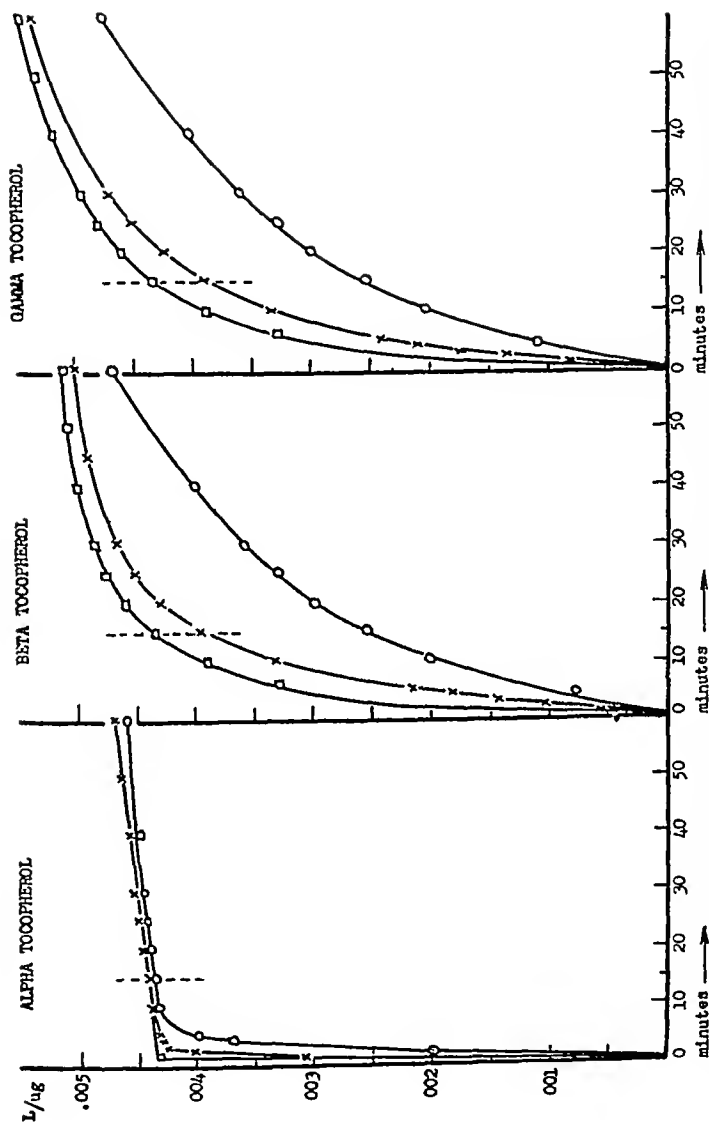


FIG 1 The effect of temperature on the rates of color development of the three tocopherols in the ferrie chloride-bipyridine system  $\square$  35°,  $\times$  27°,  $\circ$  19°. The ordinate unit,  $L$ , per microgram, represents the  $\log I_0/I$  value divided by the micrograms of tocopherol per tube (10 ml volume)

were imbedded in the slush to allow temperature adjustment. The color reagent was frozen in the refrigerator and, immediately after complete melting at room temperature, 8 ml were rapidly added to each tube. After exactly 15 minutes, less 10 seconds, the tubes were removed, rinsed quickly in cold tap water, dried with a soft cloth, and read immediately on the colorimeter. No attempt was made to correct for volume changes due to temperature in this work, although the colorimeter setting used was that obtained with the blank tube at the same temperature and time.

The standard curves of the three tocopherols are shown in Fig 2. The curves of the  $\beta$ - and  $\gamma$ -tocopherols, at both temperatures, are almost identical, and they have been so considered in the following calculations. If it were assumed that the rates of color development of the three tocopherols remained the same in mixtures as they are individually, then the 15 minutes readings obtained by treating mixtures of the three tocopherols with the color reagent, at 35° and at 15°, should allow the calculation of the  $\alpha$ -tocopherol content of a mixture according to the formula,<sup>2</sup>

$$L_{\alpha} = \frac{Z L_{15} - L_{35}}{Z - 1}$$

where  $L_{15}$  and  $L_{35}$  are the optical densities obtained on the mixture at the low and high temperature, respectively.  $Z$  is obtained from the standard curves for  $\beta$ - and  $\gamma$ -tocopherol, and it is found by dividing the  $L$  value of the 35° standard by the corresponding  $L$  value of the 15° standard for  $\beta$  and  $\gamma$ -tocopherol. After  $L_{\alpha}$  is obtained, the actual content of  $\alpha$ -tocopherol in the aliquot of the mixture is obtained from the standard  $\alpha$ -tocopherol curve.

Total tocopherol in the mixture is given by the 35° reading at 15 minutes.

The foregoing formula was tested on mixtures of standard  $\alpha$ -,  $\beta$ -, and  $\gamma$ -tocopherol solutions. The results are shown in Table I. The  $Z$  value used was 2.0. Three mixtures were used in obtaining these data. Mixture I contained 50 per cent  $\alpha$ -tocopherol of the total tocopherol, Mixture II, 47.3 per cent, and Mixture III, 64.0 per cent. The rest of the tocopherol in these mixtures consisted of equal parts of the  $\beta$  and  $\gamma$  forms. Mixtures II and III were made from a second sample of the tocopherols received 4 months after Mixture I was analyzed. New solutions were used throughout and the standard curves differed slightly.

#### *Evaluation of Total and $\alpha$ -Tocopherols in Vegetable Oils—Parker and*

<sup>2</sup> To derive this formula the basic facts used are that  $\alpha$  tocopherol gives the same reading at 35° and at 15° and that from the standard curves  $L_{35(\beta-\gamma)} = Z L_{15(\beta-\gamma)}$ . It has been assumed that in a mixture  $L_{\alpha} = L_{35} - L_{35(\beta-\gamma)}$ ,  $L_{\alpha} = L_{35} - Z L_{15(\beta-\gamma)}$ . It has also been assumed that  $L_{15(\beta-\gamma)} = L_{15} - L_{\alpha}$ . Therefore,  $L_{\alpha} = L_{35} - Z(L_{15} - L_{\alpha})$ , or  $(Z - 1)L_{\alpha} = Z L_{15} - L_{35}$ .

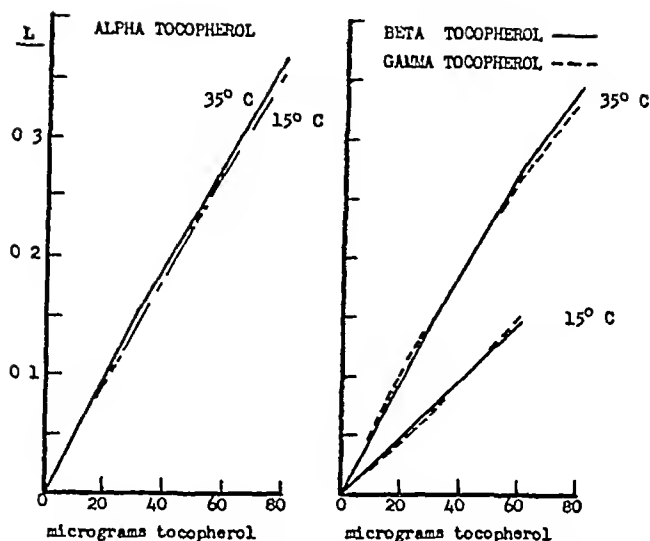


FIG 2 Standard curves of the three tocopherols at 35° and 15° The  $\log I_0/I$  value ( $L$ ) is plotted against the micrograms of tocopherol in the 10 ml reaction volume The reaction time was 15 minutes  $\pm 2$  seconds The synthetic *dl* tocopherols, as received, served as the standards

TABLE I

*Evaluation of  $\alpha$  Tocopherol in Mixtures of Standard  $\alpha$ ,  $\beta$ , and  $\gamma$  Tocopherol Solutions*

Mixture No	Tocopherol added		Tocopherol determined						
	Total	$\alpha$	Total			$\alpha$			
	$\gamma$	$\gamma$	$L_{11}$	$\gamma$	per cent recovery	$L_{11}$	$L_{12}$	$\gamma$	per cent recovery
I	26.5	13.2	0.115	24.0	90.5	0.089	0.063	12.2	92.5
	31.8	15.8	0.158	31.8	100.0	0.114	0.070	14.0	89.0
	53.0	26.3	0.248	51.5	97.2	0.189	0.130	25.8	98.2
	53.0	26.3	0.245	51.0	96.2	0.188	0.131	26.0	99.0
	79.5	40.0	0.355	77.0	97.0	0.284	0.213	43.5	109.0
	79.5	40.0	0.343	74.0	93.0	0.281	0.219	45.0	112.0
II	24.8	11.7	0.111	25.2	101.8	0.076	0.041	9.3	80.0
	49.5	23.4	0.222	50.4	101.8	0.169	0.116	27.0	115.0
	99.0	46.8	0.417	95.0	96.0	0.312	0.207	48.0	102.0
III	27.3	17.5	0.119	27.0	99.0	0.086	0.053	12.0	68.6
	54.7	35.0	0.231	52.6	96.2	0.196	0.161	36.6	104.5
	109.4	75.0	0.450	102.0	93.4	0.403	0.356	76.0	103.0

McFarlane (4) have introduced the procedure of treating 1 per cent Skelly-solve solutions of oils with 85 per cent sulfuric acid, followed by a dilute

alkali wash This effectively removes carotenoids, and it is claimed by them to remove sterols  $\alpha$ -Tocopherol is unaffected by this treatment, and this has been confirmed (3) Data have been obtained in this laboratory which show that pure solutions of  $\beta$ - and  $\gamma$ -tocopherols are also unaffected by this acid-alkali treatment, giving 100 per cent recovery

The results of such treatment of 1 per cent solutions of various vegetable oils, with and without added  $\alpha$ -tocopherol, followed by analysis for total and  $\alpha$ -tocopherols by the method outlined, are given in Table II In the

TABLE II  
*Chemical Determination of Total and  $\alpha$  Tocopherol in Vegetable Salad Oils*

Oil	Oil per 100 ml	$\alpha$ Toco- pherol added	L <sub>11</sub>	L <sub>12</sub>	Total tocopherol		$\alpha$ Tocopherol		Average total	$\alpha$ Toco- pherol of total
	gm	mg per gm oil			mg per gm oil	per cent recovery	mg per gm oil	per cent recovery	mg per gm oil	per cent
Peanut	1 12		0 042	0 030	0 36		0 16			
	1 16	0 50	0 101	0 089	0 86	100 0	0 65	99 0	0 36	62 5
	1 02		0 039	0 036	0 37		0 29			
	0 80	0 79	0 098	0 089	1 19	104 0	0 96	85 0		
Cottonseed	1 07		0 094	0 076	0 85		0 52			
	1 16	0 50	0 158	0 138	1 33	96 0	1 00	96 0	0 83	61 5
	1 02		0 083	0 065	0 80		0 50			
	0 80	0 79	0 125	0 112	1 56	96 2	1 30	101 0		
Soy bean	1 00		0 100	0 054	0 97		0 07			
	1 00	0 57	0 160	0 109	1 55	102 0	0 56	85 5		
	1 02		0 097	0 060	0 95		0 20		0 92	10 6
	0 99	0 25	0 121	0 085	1 20	100 0	0 47	107 0		
Corn	1 10		0 088	0 042	0 85		0			
	1 06	0 63	0 146	0 097	1 45	95 1	0 60	95 0		
	1 08		0 124	0 069	1 12		0 12			
	1 04	0 55	0 182	0 125	1 70	105 0	0 64	94 6	1 04	5 8
Wheat germ*	1 00		0 098	0 045	0 95		0			
	0 80	0 79	0 138	0 105	1 72	97 5	0 87	110 0		
	0 90		0 238	0 204	2 74		1 92		2 74	70 0
Whole wheat*	0 90		0 112	0 093	1 24		0 85		1 24	68 5

\* Crude Skellysolve B extracts

estimation of total tocopherol, quantitative recovery of the added  $\alpha$ -tocopherol was obtained In the evaluation of the  $\alpha$ -tocopherol (as  $\alpha$ ) content of these oils, it appeared that soy bean and corn oils contained no significant amounts The recovery of added  $\alpha$ -tocopherol (as  $\alpha$ ) was satisfactory in all cases, indicating that the soy bean and corn oils did not contain substances interfering with the evaluation of the  $\alpha$  form

*Effect of Oil Concentration on Recovery of Tocopherols*—The procedure of Parker and McFarlane (4) stipulates the use of 1 per cent oil solutions for

the acid alkali treatment and analysis, but the reason for the choice of this concentration is not given. Therefore, 2 and 4 per cent oil solutions were

TABLE III

*Determination of Total and  $\alpha$  Tocopherol in 1 Per Cent Corn Oil Solutions with Additions of  $\alpha$ ,  $\beta$ , and  $\gamma$  Tocopherols*

Addition	mg per gm oil	Total tocopherol			$\alpha$ Tocopherol*		
		$L_{11}$	mg per gm oil	per cent recovery	$L_{11}$	$L_{\alpha}$	mg per gm oil
None		0 090	1 02		0 040	0 00	0
$\alpha$ Tocopherol	1 05	0 179	2 04	97 3	0 137	0 085	0 97
$\beta$ Tocopherol	1 01	0 155	1 76	73 2	0 086	0 003	0
$\gamma$ Tocopherol	0 96	0 150	1 70	70 8	0 084	0 001	0

\* The Z factor was 1 80 for this run

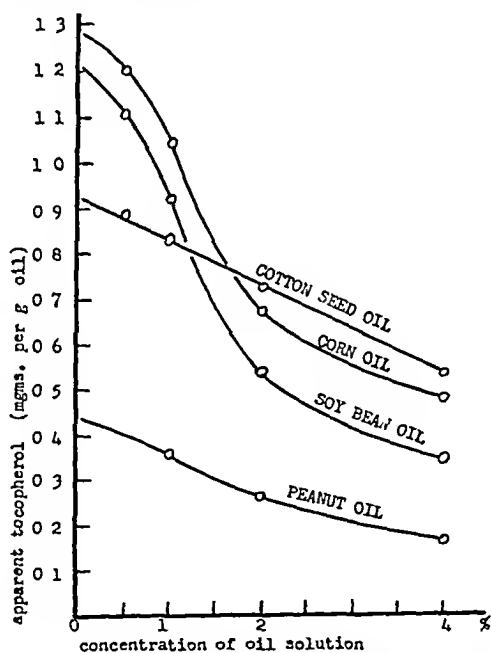


FIG 3 The effect of the concentration of oils on the apparent total tocopherol contents, after the acid alkali treatment

acid-alkali-treated, and were analyzed for total tocopherol, but with much lower results. Recoveries of added  $\alpha$ -tocopherol (as total) averaged about 80 per cent in the 2 per cent oil solutions, and about 60 per cent in the



4 per cent oil solutions As indicated in Table II, the recovery is quantitative in the 1 per cent solutions As previously indicated, the treatment of solutions of  $\beta$ - or  $\gamma$ -tocopherols with the acid and alkali resulted in no change in the rates of color development However, when  $\beta$ - or  $\gamma$ -tocopherols were added to 1 per cent oil solutions, the recovery was unsatisfactory, averaging 77.1 per cent for  $\beta$ - and 73.6 per cent for  $\gamma$ -tocopherol This was true both at 35° and at 15° Thus, the analysis of the 1 per cent oil solutions for  $\alpha$ -tocopherol (as  $\alpha$ ) is unaffected, but the total tocopherol will be more or less low, depending on the  $\beta$ - and  $\gamma$ -tocopherol content of the oils The recovery values of the three tocopherols added to corn oil are shown in Table III

The effect of the concentration of the oils on the apparent total tocopherol, as determined after the Parker-McFarlane treatment, is shown in Fig. 3 Corn and soy bean oils, which contained little  $\alpha$ -tocopherol, showed a marked increase in apparent total tocopherol upon dilution Cottonseed and peanut oils, which contained considerable amounts of  $\alpha$ -tocopherol, were much less affected by dilution Each of the points on the curves of Fig. 3 is the average of four to seven determinations When the conditions were standardized, excellent replication resulted, even at the 4 per cent oil levels The mean deviations were 2.1, 3.0, 3.2, and 4.2 per cent, for the cottonseed, corn, soy bean, and peanut oils, respectively Theoretically it is feasible to assume that the actual total tocopherol contents of the oils are given by extrapolation of the curves of Fig. 3 to zero per cent oil concentration

### DISCUSSION

The rates of color development and the stability of the color complex formed in the system, tocopherol-ferric ion-bipyridine, are highly sensitive to variations in conditions such as reagent concentration, temperature, or the presence of other substances in the system By the manipulation of conditions, principally temperature, an evaluation will be allowed of the  $\alpha$ -tocopherol content of an unknown mixture of tocopherols The method depends upon a differential between two readings made at different temperatures This indirectness will tend to lessen the accuracy of the method The data in Table I show that with an average total tocopherol of about 60  $\gamma$  per tube, the average recovery (as total) is 97.1 per cent  $\pm 2.6$  (mean deviation) With 50 per cent of the total present as  $\alpha$ -tocopherol the expected accuracy of the  $\alpha$ -tocopherol determination can be calculated from the basic formula Such a calculation shows an expected mean deviation of  $\pm 11.4$  per cent Actually, the average recovery of  $\alpha$ -tocopherol (as  $\alpha$ ) was 98.0 per cent  $\pm 10.5$  (M.D.) as shown in Table I With a smaller proportion of  $\alpha$ -tocopherol in the same amount of total tocopherol the accuracy will be still less, calculation shows that for 20 percent  $\alpha$ -tocoph

erol of the total tocopherol the expected accuracy will be  $\pm 23.4$  per cent (M D). This effect is reflected in the large variations in the values for the  $\alpha$ -tocopherol content of soy bean and corn oils which contain very small proportions of  $\alpha$ -tocopherol. However, in the absence of a more direct chemical method this procedure for  $\alpha$ -tocopherol should give valuable information.

The data show that application of the Parker-McFarlane acid-alkali treatment to 1 per cent oil solutions allows good recovery of  $\alpha$ -tocopherol, but not of  $\beta$ - or  $\gamma$ -tocopherols. The recovery of all three tocopherols is affected by the concentration of the oil. By use of several concentrations a curve of apparent total tocopherol contents of an oil can be constructed which will give, presumably, true total tocopherol by extrapolation to zero per cent oil concentration. However, it is possible that this value is due, in part, to other phenolic reducing agents. Gossypol is such a compound, and it does give a color development with the ferric ion-bipyridine color reagent at about the same rate as  $\gamma$ -tocopherol. However, gossypol is quantitatively soluble in the 85 per cent sulfuric acid, so that after the Parker-McFarlane treatment this reducing agent is eliminated and does not interfere.

Saponification of the sample in the presence of pyrogallol, as suggested by Moore and Tosie (5), was tried with little success. The  $\gamma$ -tocopherol was more resistant to destruction during saponification with or without pyrogallol. Some protection was conferred on the  $\alpha$  form, but the loss was still great.

All three tocopherols can be quantitatively adsorbed from Skellysolve onto florisil. After the solvent is removed by a stream of pure carbon dioxide, the adsorbed tocopherols are quantitatively eluted with benzene, as shown by Devlin and Mattill (3). Application of this procedure to acid-treated oil solutions gave results that differ in no significant way from those reported in Table II, either total or  $\alpha$  tocopherol.

By isolation, Emerson (6) found that of the total tocopherol in cottonseed oil, from 50 to 75 per cent is the  $\alpha$  form, the remainder being  $\gamma$ -tocopherol. This worker states that in wheat germ oil  $\alpha$ -tocopherol makes up two-thirds of the total, the rest being the  $\beta$  form. Emerson, Emerson, and Evans (7) found that corn oil contains predominantly  $\gamma$ -tocopherol and "perhaps a trace" of the  $\alpha$  form. The results obtained by the application of the method for  $\alpha$ -tocopherol, as outlined in this paper, are in agreement with these data.

#### SUMMARY

1. The effects of temperature and reagent concentrations on the rates of color development in systems containing  $\alpha$ ,  $\alpha'$ -bipyridine, ferric chloride, and pure  $\alpha$ -,  $\beta$ -, or  $\gamma$ -tocopherols have been studied.

2 A method for evaluating the  $\alpha$ -tocopherol and the total tocopherol contents of mixtures of the three tocopherols has been developed

3 This method has been applied to peanut, cottonseed, soy bean, and corn salad oils, and to crude wheat germ oil. The relative proportions of  $\alpha$ -tocopherol in these oils, as determined by this method, are in agreement with results reported from isolation work.

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# A METHOD FOR ESTIMATING TOTAL FAT SOLUBLE ANTI-OXIDANTS BASED ON THE RELATION BETWEEN FAT PEROXIDES AND CAROTENE DESTRUCTION\*

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During the course of some work on the relation between carotene destruction and the development of rancidity, it became desirable to have a rapid method for estimating total fat-soluble antioxidants. Robeson and Baxter (1) have followed antioxidant concentration of a fish liver oil by the degree of protection conferred on vitamin A alcohol in the presence of unsaturated fat at 55°. It seemed that a more adaptable method could be developed, based upon the protection of carotene against preformed fat peroxides. The various factors relating to this possibility have been investigated and are reported here.

## EXPERIMENTAL

*Relation between Peroxide Value of Fatty Materials and Destruction of Carotene, in Vitro*—The fatty material under test was peroxidized by placing it in a tall, all-glass ebullition cylinder set in a water bath at 95°. Numerous fine streams of air, which had been passed through a KOH solution, concentrated sulfuric acid, and a trap immersed in the water bath were drawn vigorously through the sample. Aliquots of the oxidizing fatty material were removed at intervals for determinations of the peroxide number by the method of Stansby (2), and of the carotene-destructive indices of the material. The latter were determined as follows. The material was accurately weighed into a 10 ml volumetric flask in quantities corresponding to about 160 gm per liter, and made up to the mark with Skellysolve E. The mixed contents were transferred to an Evelyn colorimeter tube, a blank reading taken with Filter 440, and 0.2 ml of a carotene solution in Skellysolve added. This carotene solution contained 65  $\gamma$  per ml of 90 per cent crystalline  $\beta$ -carotene (Smaco). The tube was stoppered and stored at 35° after the zero hour reading was taken. Readings were taken periodically up to 200 hours or until the carotene had been destroyed. The carotene concentrations were determined from a standard

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curve, a correction was made for the blank, and the percentage loss of carotene calculated for the various time intervals. The percentage loss of carotene was plotted against the time of storage at 35°, and from the resulting curve the time corresponding to 50 per cent loss of the carotene was noted. This is  $t_1$ . The carotene-destructive index is now obtained by multiplying  $t_1$  by the milliequivalents of peroxide per liter of the reaction solution.

TABLE I

*Relation between Carotene Destruction and Peroxide Value of Ethyl Oleate*

Time aerated at 95	Addition	Peroxide No	Carotene destructive index of oil at 35			
			Peroxide in solution	$t_1$	Index	Deviation from mean
<i>hrs</i>		<i>m eq per kg</i>	<i>m eq per l</i>	<i>hrs</i>	<i>m eq X hrs</i>	
0	None	8 0	1 1	154	169	+1
	Carotene*	7 8				
$\frac{1}{2}$	None	9 4	1 4	109	154	-14
	Carotene	9 2	1 4	103	142	-26
2	None	21 5	3 0	55 5	167	-1
	Carotene	23 8	3 3	48 5	162	-4
4	None	37 0	5 2	34 2	177	+9
	Carotene	34 7	4 9	34 3	163	-5
6	None	55 0	7 7	18 0	140	-28
	Carotene	52 3	7 3	22 0	161	-7
10	None	91 3	12 3	13 5	166	-2
	Carotene	83 4	11 2	17 0	191	+23
14	None	124	16 7	10 5	175	+7
	Carotene	95 3	13 4	13 0	174	+6
24	None	212	27 4	7 0	193	+25
	Carotene	166	21 6	9 0	194	+26
48	None	456	64 0	2 4	154	-14
	Carotene	465	65 1	2 7	176	+8
Average					168	±12

\* 15  $\gamma$  of  $\beta$ -carotene were added per gm of oil

The results obtained on ethyl oleate (Eastman) are given in Table I. Peroxidation was carried out on the ester alone, and on the ester plus the addition of 15  $\gamma$  of carotene per gm. The added carotene was destroyed within the 1st hour of aeration, the oxidation products of this carotene appeared to have no influence on the subsequent determinations of the carotene-destructive indices. It is evident from Table I that, under the conditions employed, the destruction of carotene at 35° is closely correlated to oleate-peroxide concentration, the average carotene-destructive index is 168.

A few runs were made on the more unsaturated fat acid esters. Methyl linolate samples with peroxide numbers of 130, 290, and 450 milliequivalents per kilo showed carotene-destructive indices of 130, 109, and 122, respectively, with an average of 120. A methyl linolenate sample of peroxide number 421 gave a value of 111. While these values are too few to be significant, they do indicate that the indices of all three unsaturated fat acids are roughly in the same general order of magnitude.

Similar procedures have been carried out on edible soy bean oil, peanut oil, and cottonseed oil and the results summarized in Table II. In general, it is apparent from these data that the carotene destructive indices of the various natural oils tend to approach the same order of magnitude as was

TABLE II

*Relation between Carotene Destruction and Peroxide Value of Edible Vegetable Salad Oils Aerated at 95°*

	Cottonseed oil	Peanut oil	Soy bean oil
No. of determinations	6	6	6
Carotene destructive indices	129 $\pm$ 10*	128 $\pm$ 11*	157 $\pm$ 12*
Peroxide No., range	190-341	57-275	197-246
Hrs. aerated for same	26-48	23-45	35-48
Highest peroxide No. showing no carotene destruction in 200 hrs. (index >2000)	20	17	75
Hrs. aerated for same	8	10	11
Total tocopherol at this time mg per gm oil	0.19	0.10	0.35
Original total tocopherol, mg per gm	0.79	0.34	0.97

\*Mean deviation

obtained on the fat acid esters. However, this level is reached only at high peroxide values or after the peroxide peak has been passed. At lower peroxide values the destruction of carotene proceeds very much more slowly than might be expected. The simplest explanation of this is that the naturally occurring antioxidants are not completely destroyed until quite high peroxide levels have been reached. This is corroborated by the results of total tocopherol determinations (3), as shown in Table II. Golumbic (4) has noted the formation of a red quinoid type antioxidant formed during oxidation of vegetable fats. This was similar to, but not identical with, a compound formed from  $\alpha$ -tocopherol. He did not compare it with the  $\gamma$ -tocophenone. This may account for part of the residual antioxidants noted.

The data in Tables I and II have shown the degree of correlation between

carotene destruction and peroxide concentration in a system in which the oil concentration was constant and variations in the peroxide concentrations were obtained by using oils of varying states of oxidation

The next point to establish was the effect of variations in the oil concentration of the system. Ethyl oleate, peanut oil, and soy bean oil were peroxidized to about 250 milliequivalents per kilo. Solutions of these oils in Skellysolve E were diluted to give various oil concentrations, and the determinations of the carotene-destructive indices carried out as before

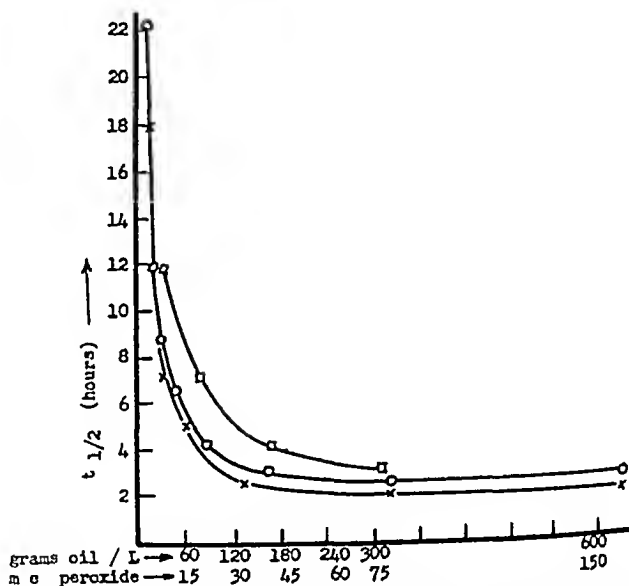


FIG 1 The relation between concentration of oils, peroxidized to 250 milliequivalents per kilo, and the time ( $t_{1/2}$ ) for half loss of carotene, originally at 12 mg per liter. The temperature was 35°. O, peanut oil, X, ethyl oleate, □, soy bean oil

The  $t_{1/2}$  values are shown in Fig 1. It is apparent that the proportionality between peroxide and carotene destruction breaks down at the higher fat concentrations. An oil concentration of 160 gm per liter was the highest that could be used efficiently. At this concentration the peroxidized peanut oil was completely colorless and gave no blank reading when compared with pure solvent. The other oils did show more or less color and required blank corrections. For this reason peanut oil appeared to be most adaptable as the source of peroxides for the method in mind.

The effect of variations in the carotene concentration in the system com

posed of 160 gm of the peroxidized peanut or soy bean oils per liter in Skellysolve E is shown in Fig 2. From these data it is evident that, although the original carotene concentration is somewhat critical, slight variations in amount will have an insignificant bearing on the course of the reaction. For ease and speed the concentration of 1.2 mg of carotene per liter was adopted for the proposed method.

*Antioxidant Activity in Protecting Carotene against Fat Peroxides*—The highly peroxidized oil was made up in Skellysolve E in a concentration such

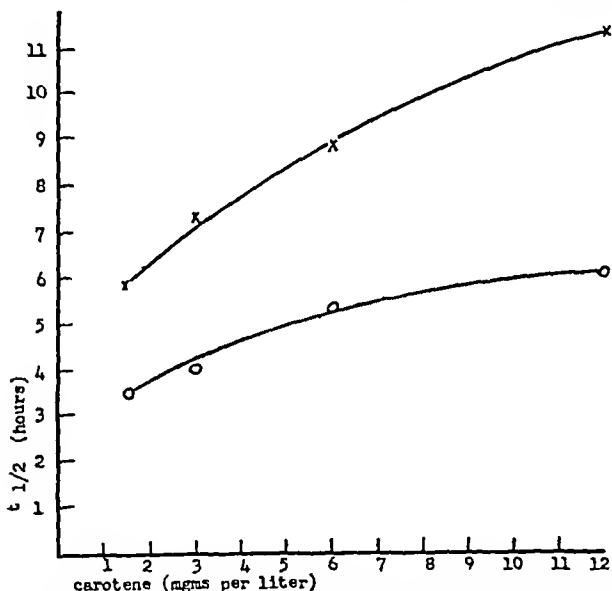


FIG 2 The relation between the concentration of carotene and the time ( $t_1$ ) for half loss of the carotene in the presence of peroxidized peanut oil (O), or peroxidized soy bean oil (X). The temperature was 35°, and the oil concentrations were 160 gm per liter.

that 5 ml of the solution would contain the quantity of oil desired in 10 ml of the final reaction mixture. This was 320 gm of peroxidized peanut oil per liter. The 5 ml of this solution were placed in an Evelyn colorimeter tube, followed by 5 ml of a Skellysolve E solution of the antioxidant to be tested, 0.2 ml of the carotene solution was added to give a final carotene concentration of about 1.2  $\gamma$  per ml. After the zero hour reading on the Evelyn colorimeter was taken, the tubes were stored at 35° and readings made at intervals. The  $t_1$  values were obtained from these readings ac-



according to the procedure described previously. The activities of the various antioxidants were determined by the expression

$$\frac{t_x - t_0}{t_0} \frac{1}{\text{micrograms antioxidant}} = K$$

where  $t_x$  is the  $t_1$  of the tube containing the antioxidant under test and  $t_0$  is the  $t_1$  of the tube containing no antioxidant.

The carotene-protecting action of  $\alpha$ -tocopherol added to the above described system was determined. With levels varying from 5 to 100  $\gamma$

TABLE III

*Effect of Age of Peroxidized Peanut Oil Solution on Carotene Destruction and Protective Action of  $\alpha$ -Tocopherol*

Age before use*	Addition per 10 ml reaction solution†		$t_1$	$\frac{t_x - t_0}{t_0}$	K
days		$\gamma$	hrs		
0	None		4 20		
	$\alpha$ -Tocopherol	8 9	5 95	0 417	0 0470
1	"	17 8	7 68	0 830	0 0467
	None		3 55		
6	$\alpha$ -Tocopherol	17 8	6 63	0 868	0 0487
	None		3 08		
9	$\alpha$ -Tocopherol	8 9	4 45	0 445	0 0500
	"	17 8	5 65	0 835	0 0470
14	None		3 02		
	$\alpha$ -Tocopherol	8 9	4 38	0 450	0 0505
15	"	17 8	5 64	0 868	0 0487
	None		2 88		
50	$\alpha$ -Tocopherol	17 8	5 20	0 806	0 0454
	None		2 80		
50	$\alpha$ -Tocopherol	17 8	4 90	0 750	0 0420
	None		1 97		
50	$\alpha$ -Tocopherol	30 4	4 25	1 17	0 0385
	None				

\* Stored at 10°

† Determinations run at 35°

of tocopherol per tube, eleven values for  $K$  showed a mean deviation of  $\pm 8$  per cent. In this range the antioxidant activity stands in linear relationship with the original concentration of  $\alpha$ -tocopherol.

The effect of age of a stock solution of the peroxidized peanut oil on the  $K$  values for  $\alpha$ -tocopherol is shown in Table III. It is apparent that the  $t_0$  value decreased as the oil aged, undoubtedly owing to continued oxidation of the oil, but that the  $K$  value for the tocopherol showed no serious change up to 2 weeks. The seven values obtained prior to this time showed a mean deviation of  $\pm 2.3$  per cent.

The  $k$  values of  $\alpha$ -,  $\beta$ -, and  $\gamma$ -tocopherols,<sup>1</sup> gossypol, dianilinogossypol, lecithin, and cholesterol in the fat peroxide carotene system are given in Table IV. There is little difference in activities of the three tocopherols. Gossypol and dianilinogossypol, prepared according to the methods of Campbell, Morris, and Adams (5), will be found to have equal activity if the values in Table IV are converted to a molecular basis, and are equal to the tocopherol value. Fat-free lecithin and cholesterol possess no anti-

TABLE IV

*Activity of Pure Antioxidants in Inhibiting Carotene Destruction by Preformed Peanut Oil Peroxides*

Addition to 10 ml reaction solution		$t_1$	$\frac{t_2 - t_0}{t_0}$	$K$
	$\gamma$	hrs		
None		2 70		
$\alpha$ -Tocopherol	9 0	3 92	0 452	0 0502
"	18 0	5 00	0 850	0 0475
$\beta$ -Tocopherol	8 4	3 80	0 408	0 0500
"	16 8	5 04	0 864	0 0514
$\gamma$ -Tocopherol	11 3	4 52	0 675	0 0597
"	22 6	6 05	1 24	0 0550
Gossypol	23 0	5 62	1 08	0 0470
"	30 0	6 27	1 32	0 0440
"	40 0	7 70	1 85	0 0462
Dianilinogossypol	12 6	3 80	0 408	0 0323
"	25 2	5 20	0 920	0 0365
None		2 88		
Cholesterol	1000	2 90		
Lecithin	100	2 90		
"	200	2 85		
"	400	2 70		
"	800	2 65		
$\alpha$ -Tocopherol	18 0	5 20	0 810	0 0455
" + lecithin	100	5 07	0 755	0 0420
" + "	200	4 95	0 704	0 0397
" + "	400	4 75	0 650	0 0365
" + "	800	4 45	0 545	0 0312

oxidant activity. Combinations of  $\alpha$ -tocopherol and lecithin did not show an increased activity. In fact, the opposite was true, large amounts of lecithin tended to repress the activity.

*Application of Carotene Protection Method to Estimation of Total Antioxidants in Vegetable Oils*—The oil to be analyzed was made up in Skelly-

<sup>1</sup> The supplies of the synthetic tocopherols were furnished by Merck and Company, Inc.

solve E and appropriate amounts of this sample added to 5 ml of the peroxidized peanut oil stock solution in Evelyn colorimeter tubes. Sufficient Skellysolve E had previously been added to make 10 ml final volumes, 0.2 ml of the carotene solution was added, mixed, and the readings taken as described before. The  $(I_x - I_0)/I_0$  values obtained from these determinations were divided by the  $K$  value of mixtures of equal parts of the three

TABLE V

*Estimation of Total Antioxidant in Vegetable Salad Oils by Carotene Protection Method*

Oil analyzed	Sample	$\frac{I_x - I_0}{I_0}$	Antioxidant as tocopherol		
			In sample	In oil	Average
	gm		$\gamma$	mg per gm	mg per gm
Peanut	0.021	0.35	6.7	0.33	0.38 $\pm$ 0.025*
	0.052	1.07	20.5	0.39	
	0.103	2.20	42.3	0.41	
	0.155	3.00	57.5	0.39	
Cottonseed	0.02	0.96	18.3	0.91	0.93 $\pm$ 0.023*
	0.03	1.45	27.7	0.92	
	0.04	2.03	39.0	0.97	
	0.05	2.40	46.1	0.91	
Soy bean	0.002	0.10	2.0	1.00	1.08 $\pm$ 0.038*
	0.004	0.22	4.3	1.07	
	0.010	0.57	11.2	1.11	
	0.020	1.18	23.1	1.14	
	0.040	2.25	43.0	1.07	
	0.004†	1.15	22.5		
Corn	0.002	0.12	2.4	1.19	1.19 $\pm$ 0.043*
	0.004	0.25	4.9	1.17	
	0.011	0.60	11.8	1.12	
	0.021	1.37	26.8	1.26	
	0.031	2.04	39.0	1.25	
	0.040	2.42	46.0	1.15	
	0.004†	1.17	23.0		

\* Mean deviation

† Plus 18  $\gamma$  of tocopherol

tocopherols. This gave the antioxidant, expressed as tocopherol, per aliquot of the oil added. This quantity divided by the weight of the sample gave antioxidant in mg per gm of the oil.

The results of series of such analyses on peanut, cottonseed, soy bean, and corn salad oils, and on a crude wheat germ oil are given in Table V. The  $K$  value of 0.0522 was used in the majority of cases. Some of the values on the lower levels of soy bean and corn oil were obtained on another day's run, and a  $K$  value of 0.0510 was used. It has been the writers'

practice to set up standards with every run, and to use the resultant *K* value for that run only. It will be noted from Table V that the recovery of tocopherol added to soy bean or corn oils was good. The results obtained on any one oil show a mean deviation of less than  $\pm 7$  per cent. The antioxidant contents of the oils were only slightly higher than the total tocopherol contents of the same oils determined by the non-bipyridine method (3). Thus it appears that these salad oils contain little or no antioxidants other than those that give the iron bipyridine test.

*Application of Carotene Protection Method to Estimation of Total Antioxidants in Biological Materials*—The method for total antioxidant would be most useful when applied to natural materials. The extractive techniques and a few results on dry yeast and rat liver are reported here.

A sample of dry yeast (Northwestern brand) was thoroughly extracted at room temperature with a mixture of 2 parts of ethanol and 1 part of Skellysolve B. Water was added to the extract to give a 60 per cent alcohol solution. This was separated and washed with Skellysolve B. The combined Skellysolve fractions were washed with water, dried with sodium sulfate, and made up to a convenient volume. The yeast contained 13 per cent of Skellysolve B-soluble material under these extractant conditions. Six determinations on this extract varied from 2.38 to 2.65 mg of antioxidant, expressed as tocopherol, per gm of yeast fat, with an average of 2.5 mg per gm, and a mean deviation of  $\pm 3.4$  per cent. However, the average recovery of added  $\alpha$ -tocopherol was only 86.1 per cent. This low recovery may be related to the adverse effect of lecithin or related substances as noted in Table IV.

The presence in yeast of a fat-soluble antioxidant was further demonstrated by organoleptic keeping quality tests. The extracted yeast residue, mixed with 10 per cent lard, became rancid after 9 days storage, the original yeast mixed with 10 per cent lard was still sweet after 50 days storage at 80°. A control mixture of corn-starch with 10 per cent lard became rancid in 1 day. György and Tomarelli (6) have noted the antioxidant activity of natural sources of the B vitamins, including yeast. They believe this antioxidant to be water-soluble. However, they did not exclude the possible complicity of fat-soluble antioxidants.

Similar extraction techniques were applied to the livers (fresh) of four rats, and the antioxidant content of the Skellysolve extracts determined. One rat had been on a diet low in vitamins A and E, the second rat had been on the same diet but received 2  $\gamma$  of carotene daily, the third rat had been on the same diet but received the carotene and 0.8 mg of  $\alpha$ -tocopherol daily, while the fourth rat had been on a stock diet. The rats had been on their respective diets for 2 to 4 months since weaning. The average results for the antioxidant concentration in the livers of these rats were

solve E and appropriate amounts of this sample added to 5 ml of the peroxidized peanut oil stock solution in Evelyn colorimeter tubes. Sufficient Skellysolve E had previously been added to make 10 ml final volumes, 0.2 ml of the carotene solution was added, mixed, and the readings taken as described before. The  $(t_x - t_0)/t_0$  values obtained from these determinations were divided by the  $K$  value of mixtures of equal parts of the three

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	0.052	1.07	20.5	0.39	
	0.103	2.20	42.3	0.41	
	0.155	3.00	57.5	0.39	
Cottonseed	0.02	0.96	18.3	0.91	$0.93 \pm 0.023^*$
	0.03	1.45	27.7	0.92	
	0.04	2.03	39.0	0.97	
	0.05	2.40	46.1	0.91	
Soy bean	0.002	0.10	2.0	1.00	$1.08 \pm 0.038^*$
	0.004	0.22	4.3	1.07	
	0.010	0.57	11.2	1.11	
	0.020	1.18	23.1	1.14	
	0.040	2.25	43.0	1.07	
Corn	0.004†	1.15	22.5		$1.19 \pm 0.043^*$
	0.002	0.12	2.4	1.19	
	0.004	0.25	4.9	1.17	
	0.011	0.60	11.8	1.12	
	0.021	1.37	26.8	1.26	
	0.031	2.04	39.0	1.25	
	0.040	2.42	46.0	1.15	
	0.004†	1.17	23.0		

\* Mean deviation

† Plus 18  $\gamma$  of tocopherol

tocopherols. This gave the antioxidant, expressed as tocopherol, per aliquot of the oil added. This quantity divided by the weight of the sample gave antioxidant in mg per gm of the oil.

The results of series of such analyses on peanut, cottonseed, soy bean, and corn salad oils, and on a crude wheat germ oil are given in Table V. The  $K$  value of 0.0522 was used in the majority of cases. Some of the values on the lower levels of soy bean and corn oil were obtained on another day's run, and a  $K$  value of 0.0510 was used. It has been the writers'

2 Aeration of edible vegetable oils at 95° revealed a lack of correlation between peroxide value and subsequent carotene destruction at 35° until quite high peroxide numbers were reached. Evidence was presented to show that this may be due to the persistence of natural antioxidants in the oxidizing oils. After complete loss of antioxidants, the carotene-destructive indices of the oxidizing oils approached the same order of magnitude as was obtained with the pure unsaturated fat acid esters.

3  $\alpha$ -,  $\beta$ -, and  $\gamma$ -tocopherols or mixtures of the three, and gossypol were essentially equivalent in protecting carotene against preformed fat peroxides. Lecithin and cholesterol were without activity as antioxidants.

4 A method was developed for estimating total fat-soluble antioxidants based upon the protection of carotene against preformed fat peroxides. This method was applied to vegetable salad oils, dry yeast, and rat liver.

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# THE EFFECT OF TEMPERATURE ON THE RELATIVE ANTI OXIDANT ACTIVITY OF $\alpha$ -, $\beta$ -, AND $\gamma$ -TOCOPHEROLS AND OF GOSSYPOL\*

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Olcott and Emerson (1) found the antioxidant activity of  $\alpha$ -,  $\beta$ -, and  $\gamma$ -tocopherols to be in the ratio of 1:17:43, respectively, for the protection of lard against peroxide formation at 75°. This is the reverse order of the vitamin activity of these substances. Hickman, Harris, and Woodside (2) found that the three tocopherols were of equal potency in what they term the co-vitamin activity of providing protection to carotene or vitamin A against destruction in the gastrointestinal tract of rats. Smith, Spillane, and Kolthoff (3) have observed that  $\beta$ - and  $\gamma$ -tocopherols are oxidized at a more positive potential than is  $\alpha$ -tocopherol. This indicates that the  $\alpha$  form has the greatest antioxygenic capacity, and under proper conditions should prove to be the best antioxidant of the three tocopherols.

The conditions of the first two cited experiments differ in three major respects. The temperature is 75° in one case, and body temperature in the other. The substance protected is unsaturated carbon bonds in the one experiment, and carotene in the other, and the oxidizing agent is atmospheric oxygen, as compared with active fat acid peroxides presumably formed by some agent in rat stomach mucosa (4).

In an attempt to obtain an explanation for the observed variations in the relative antioxidant activities of the three tocopherols, experiments were set up to determine the relative activity of antioxidants at various temperatures. Two systems were studied: the protection of carotene in the presence of an excess of fat peroxides, and the protection offered against the development of peroxides during aeration of an unsaturated fat acid ester.

## EXPERIMENTAL

*Protection of Carotene against Fat Peroxides*—The bases of the methods used have been outlined in a previous paper (5). In brief, the procedure consists of pipetting 5 ml. of a solution of peroxidized peanut oil (320 gm. per liter in Skellysolve F) into an Evelyn colorimeter tube, followed by 5

\* Published with the approval of the Director of the Alabama Agricultural Experiment Station. This investigation was aided by a grant from the Nutrition Foundation, Inc.



ml of Skellysolve E or a Skellysolve E solution of the antioxidant to be tested, 0.2 ml of a carotene solution (65  $\gamma$  of 90 per cent crystalline  $\beta$  carotene (Smaco) per ml) is added, and the tube is stoppered and mixed. The zero hour readings are taken on the Evelyn colorimeter (Filter 440) and the tubes are stored at the desired temperature. Readings are taken at intervals thereafter. Carotene concentrations are obtained from a standard curve and the percentage loss of carotene calculated. This percentage loss is plotted against time of storage. From the resultant curve, the time corresponding with 50 per cent loss of carotene is noted. This

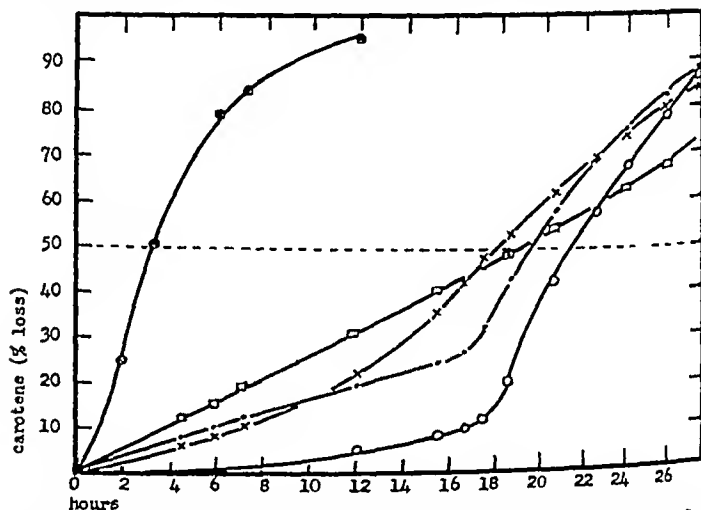


FIG. 1 The rates of carotene destruction by peroxidized peanut oil at 30°, in the presence of various antioxidants.  $\oplus$ , no antioxidant added,  $\circ$ , 91  $\gamma$  of  $\alpha$ -tocopherol,  $K$  is 0.0594,  $\bullet$ , 84  $\gamma$  of  $\beta$ -tocopherol,  $K$  is 0.0576,  $\times$ , 71  $\gamma$  of  $\gamma$ -tocopherol,  $K$  is 0.0626,  $\square$ , 80  $\gamma$  of gossypol,  $K$  is 0.0589.

time period is represented as  $t_{\frac{1}{2}}$ . The activities,  $K$ , of the antioxidants are determined from the expression

$$\frac{t_x - t_0}{t_0} \frac{1}{\text{micrograms antioxidant}} = K$$

where  $t_x$  and  $t_0$  are the  $t_{\frac{1}{2}}$  values of the tubes containing the antioxidant, without antioxidant, respectively.

The rates of carotene destruction in the presence of tocopherols<sup>1</sup> and gossypol at 30° are given in Fig. 1. Calculations made according to the

<sup>1</sup> The supplies of the synthetic tocopherols were furnished by Merck and Company, Inc.

foregoing formula indicate that the  $K$  values, based upon the carotene half life, of the four antioxidants are approximately of the same order of magnitude. However, the nature of the curves differs radically.  $\alpha$ -Tocopherol possesses a well defined induction period during which little carotene is destroyed.  $\beta$ -Tocopherol has a much less well defined induction period, and it allows considerable carotene destruction before the break in

TABLE I

*Effect of Temperature on Antioxidant Activities of  $\alpha$ -,  $\beta$ -, and  $\gamma$ -Tocopherols in Fat Peroxide-Carotene System*

Temperature	Tocopherol added		$t_{\frac{1}{2}}$	$\frac{t_x - t_0}{t_0}$	$K$	Relative activity
<i>C</i>		<i><math>\gamma</math></i>	<i>hrs</i>			
4	None		27.0			
	$\alpha$ -	14.0	62.8	1.33	0.095	1
	$\beta$ -	16.2	63.8	1.36	0.084	0.88
	$\gamma$ -	15.3	67.5	1.50	0.098	1.03
25	None		5.2			
	$\alpha$ -	21.0	11.6	1.22	0.0581	1
	$\beta$ -	20.2	10.9	1.07	0.0523	0.90
	$\gamma$ -	19.1	12.0	1.31	0.0685	1.17
35	None		2.6			
	$\alpha$ -	700.0	93.7	35.0	0.0500	1
	$\beta$ -	606.0	84.1	31.3	0.0516	1.03
	$\gamma$ -	546.0	95.2	35.5	0.0650	1.30
58	None		1.2			
	$\alpha$ -	700.0	13.3	10.0	0.0143	1
	$\beta$ -	606.0	13.1	9.9	0.0164	1.15
	$\gamma$ -	546.0	15.5	14.3	0.0218	1.52
			<i>min</i>			
82	None		7.0			
	$\alpha$ -	700.0	45.5	5.50	0.0079	1
	$\beta$ -	606.0	48.0	5.86	0.0097	1.22
	$\gamma$ -	546.0	64.0	8.14	0.0149	1.89
98	None		2.7			
	$\alpha$ -	700.0	13.4	3.96	0.0056	1
	$\beta$ -	606.0	13.5	4.00	0.0067	1.23
	$\gamma$ -	546.0	22.5	7.34	0.0134	2.40

the curve. The  $\gamma$ -tocopherol has even less of a break in the curve, while gossypol has none. Obviously, induction periods are meaningless as a measure of antioxidant activity under these conditions.

Although the periods of half life of carotene indicate that these antioxidants are of approximately equal activity under these conditions, the rates of loss of carotene prior to the half loss time demonstrate clearly that

$\alpha$ -tocopherol has the greatest antioxidant potency and is followed in order by  $\beta$ - and  $\gamma$ -tocopherol and gossypol. These results demonstrate the distinction that should be made between the activity and the potency of an antioxidant, the activity of an antioxidant represents a general quantitative expression, the value of which depends upon the characteristics of all ingredients in the system as well as the criterion used as the end point, while the potency of an antioxidant is a more absolute value which probably is represented by its oxidation-reduction potential.

The effect of temperature upon the relative antioxidant activities of the three tocopherols in the fat peroxide-carotene system is shown in Table I. Increasing temperatures resulted in decreasing antioxidant activity in all cases but more so in the case of the  $\alpha$ -tocopherol. This resulted in an apparently greater relative activity of the  $\beta$ -, and especially, the  $\gamma$  tocopherols.  $\gamma$ -Tocopherol increased 133 per cent in activity relative to  $\alpha$  tocopherol between 4-98°, while  $\beta$ -tocopherol increased 40 per cent. Five determinations at 98-100° resulted in average  $K$  values for  $\alpha$ -,  $\beta$ -, and  $\gamma$  tocopherols of 0.0046, 0.0055, and 0.0110, with mean deviations of  $\pm 11$ ,  $\pm 9$ , and  $\pm 12$  per cent, respectively. However, the mean deviations from the average *relative activity* values were less.  $\beta$ -Tocopherol averaged 1.23 times more active than  $\alpha$ -tocopherol, and showed a mean deviation of  $\pm 2.5$  per cent,  $\gamma$ -tocopherol averaged 2.42 times more active than  $\alpha$  tocopherol, and showed a mean deviation of  $\pm 6.4$  per cent. These results were obtained with from 0.5 to 3.0 mg. of the tocopherols per tube.

The rates of destruction of the tocopherols and carotene in the fat-peroxide system at 35°, 58°, and 98° are shown in Fig. 2. The tocopherol terminations were made by removing 1 ml. aliquots at intervals from basal tubes run in the usual way at the desired temperature. The tocopherol contents of these 1 ml. aliquots were determined by placing them in Evelyn colorimeter tubes containing 4.5 ml. of the peroxidized peanut oil stock solution and 4.5 ml. of Skellysolve E. Carotene was added to these determinative tubes in amounts to give a total of 12.5  $\gamma$  per tube. These tubes were then stored at 35° and the  $t_1$  values obtained in the usual way (5). The tocopherol contents of the determinative tubes, and therefore of the 1 ml. aliquots, were obtained by comparison with similar tubes containing known amounts of the tocopherol standards. Controls were run by similar treatment of 1 ml. aliquots taken from basal tubes run at the temperature of the experiment, but containing no antioxidants. The results showed that the heat treatments to which the 1 ml. aliquots from the basal tubes had been subjected did not influence the subsequent determinative assays.

The data presented in Fig. 2 may furnish an explanation of the great effect of temperature on the relative antioxidant activities of the tocopherols

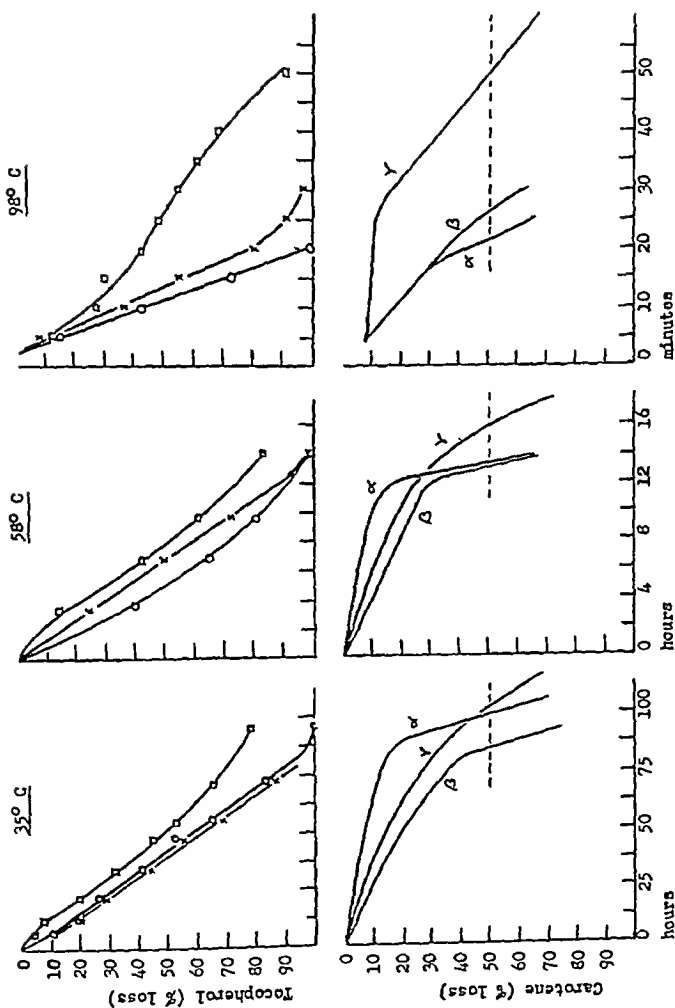


Fig. 2 The rates of loss of the tocopherols and of carotene, at 35°, 58°, and 98°, in the presence of peroxidized peanut oil. O,  $\alpha$ -tocopherol; X,  $\beta$ -tocopherol;  $\square$ ,  $\gamma$ -tocopherol.

points of difference. In the aerated ethyl oleate system, the  $K$  values appear to possess temperature optima rather than show a continuous falling off in value with increased temperature, such as was the case in the peroxide-carotene system. Gossypol and red gossypol are of about equal effectiveness in preventing peroxide development, and show a temperature variation that is similar to that of the  $\alpha$ -tocopherol.

The antioxidant property of gossypol was first shown by Mattill (8) in studies on the keeping quality of lard at elevated temperatures. He also

TABLE III  
*Effect of Temperature on Antioxidant Activity in Aerated Ethyl Oleate*

Temperature	Antioxidant added to oil		End point*	$\frac{t_x - t_0}{t_0}$	$K$	Relative activity
$^{\circ}\text{C}$		mg per gm	days			
35	None		5.7			
	$\alpha$ -Tocopherol	0.0445	10.3	0.81	18.2	1
	$\beta$ -Tocopherol	0.0500	9.8	0.72	14.4	0.79
	$\gamma$ -Tocopherol	0.0355	14.0	1.45	17.0	0.94
	Gossypol	0.0460	11.9	1.09	23.7	1.30
			hrs			
60	None		4.8			
	$\alpha$ -Tocopherol	0.20	25.8	4.38	21.9	1
	$\beta$ -Tocopherol	0.20	32.4	5.75	28.7	1.31
	$\gamma$ -Tocopherol	0.20	41.9	7.72	38.5	1.76
	Gossypol	0.20	35.9	6.50	32.4	1.47
	Red gossypol	0.20	34.8	6.30	31.5	1.44
	Dianilinogossypol	0.20	32.0	5.67	28.3	1.29
95	None		1.5			
	$\alpha$ -Tocopherol	0.50	4.4	1.93	3.8	1
	$\beta$ -Tocopherol	0.50	9.5	5.34	10.0	2.63
	$\gamma$ -Tocopherol	0.45	14.5	8.70	19.3	5.10
	Gossypol	0.20	3.5	1.33	6.7	1.76
	Red gossypol	0.20	3.6	1.42	7.1	1.87
	Dianilinogossypol	0.20	4.4	1.95	9.8	2.68

\* The end-point was the time ( $t$ ) required for an increase of 20 milliequivalents of peroxide per kilo of the ethyl oleate.

demonstrated that gossypol acetate had no antioxidant effect. The results in Table III show that dianilinogossypol is at least equal to the free gossypol, on a molecular basis. This indicates that the aldehyde groups of gossypol are not essential to its antioxidant property.

#### DISCUSSION

The condition of temperature has received little attention in studies on the effectiveness of antioxidants in fat-soluble systems. In the food

industry, keeping quality tests of fats and other foods are almost universally carried out at elevated temperature on the general assumption that such results can be transposed to normal storage temperatures. The data presented in this paper show that the relative antioxidant activities of the three known naturally occurring tocopherols are markedly affected by temperature, under the conditions employed. It is possible that this temperature factor will operate in a similar manner, even in the more complicated systems present in food products. Qualitatively, the same effect of temperature on the antioxidant activities of the tocopherols has been obtained in both the fat peroxide-carotene system, and in the aerated ethyl oleate system, indicating a general condition, since these two systems differ markedly.

The temperature effect on the relative antioxidant activity of the tocopherols may be related to differences in stability or to differences in antioxidant potencies at the various temperatures. The method used for following the rate of tocopherol loss actually measures antioxidant and not necessarily unaltered tocopherol. It is possible that the first oxidation products of the tocopherols, i.e. the tocoquinones, possess antioxidant activity and are entering into the system. This would explain the fact that at the higher temperatures the  $\gamma$ -tocopherol appears to be destroyed in two steps. The  $\gamma$ -tocoquinone may be more stable or more effective in establishing antioxidant relations than the corresponding  $\alpha$ - or  $\beta$ -quinones, thus explaining the much greater apparent antioxidant activity of the  $\gamma$ -tocopherol at high temperatures. Some such assumption must be made, since the oxidation-reduction potentials of the pure, unaltered  $\beta$ - and  $\gamma$ -tocopherols are practically identical, as shown by either the polarograph (3), or by the rates of reaction with ferric ions in the Emmerie-Engel test (9). It is known that this color reagent does not react with the tocoquinones.

The carotene destruction curves in the presence of the various antioxidants (Fig. 1) show that, with the criterion used, the activities (at 30°) of the three tocopherols are about the same. However, if the degree of protection prior to the point of 50 per cent destruction of carotene is considered, these curves show clearly that  $\alpha$  tocopherol is the most potent, followed by  $\beta$ - and  $\gamma$ -tocopherol. These data may have physiological significance, since they show a variation in tocopherol potencies which are in line with the known vitamin E activity and under conditions that are not physiologically impossible. Normal tissue contains the highly unsaturated fat acids and it has been shown, *in vitro*, that these can be peroxidized by hemoglobin or related compounds with the destruction of the heme molecule (10). Water extracts of minced tissues have been shown (4) to destroy carotene in the presence of linolate, this reaction is inhibited

by  $\alpha$  tocopherol    Presumably, vitamin A will react the same as carotene in such systems

#### SUMMARY

1 The relative antioxidant activity of  $\alpha$ -,  $\beta$ -, and  $\gamma$ -tocopherols has been found to be dependent upon temperature. At low temperatures the three compounds showed approximately equal activity, while at elevated temperatures the  $\gamma$ -tocopherol was several times more active than the  $\alpha$  form. This held true both for the protection of carotene against preformed fat peroxides and for the inhibition of peroxide formation in aerated ethyl oleate.

2 Gossypol was about equal to the tocopherols as an antioxidant at 35°. The dianilnogossypol was equal to the free gossypol on a molecular basis.

3 A distinction is pointed out between antioxidant *potency* and *activity*. Evidence was obtained that at physiological temperatures  $\alpha$  tocopherol had the greatest antioxygenic *potency*, followed in order by  $\beta$ - and  $\gamma$  tocopherols and gossypol.

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# GOSSYPOL AS A CAROTENE-PROTECTING ANTIOXIDANT, IN VIVO AND IN VITRO\*

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Sherman (1) has shown that carotene is destroyed in the gastrointestinal tract when fed with linolate or linolenate to vitamin A-deficient rats. Carotene-free refined vegetable oils or their non-saponifiable fractions prevented this destruction of carotene. It was shown later (2) that  $\alpha$ -tocopherol was highly effective in preserving carotene under such conditions. This fact was noted independently by Quaekenbush, Cox, and Steenbock (3). These results have been confirmed by Hickman and coworkers (4-6), and extended in detail to show that  $\beta$ - or  $\gamma$ -tocopherols or fat-soluble derivatives of ascorbic acid and hydroquinone are equally as effective as  $\alpha$ -tocopherol.

The antioxidant property of gossypol for the protection of lard, *in vitro*, was first demonstrated by Mattill (7). Gossypol is effective in protecting carotene, *in vitro*, against preformed fat peroxides (8). Therefore, it seemed logical to determine whether gossypol could act as a carotene-protecting antioxidant, *in vivo*, and whether cottonseed products containing gossypol could inhibit carotene destruction and rancidity development, *in vitro*.

## EXPERIMENTAL

*Carotene Protection, in Vivo*—The procedure used by previous workers has been to deplete rats on the U S P vitamin A-low diet, or modifications thereof. When body weights have plateaued, small amounts of carotene or other vitamin A-active materials were fed daily along with the antioxidant to be tested. The resultant growth rate is the criterion for effectiveness of the antioxidant.

*Ration*—The U S P vitamin A-low basal diet contains 8 per cent of dry yeast as the source of water-soluble vitamins. It was found in this laboratory (8) that the 1.3 per cent of crude fat in the dry yeast is rich in some fat-soluble antioxidant. At the 8 per cent level, therefore, the yeast would contribute 0.1 gm of crude fat and 30  $\gamma$  of antioxidant, expressed as tocopherol, to 100 gm of the basal diet. Because of the presence of these materials, yeast was omitted from the ration for the experiments.

\* Published with the approval of the Director of the Alabama Agricultural Experiment Station.



reported in this paper and the water-soluble vitamins were supplied as the synthetic compounds. The diet, indicated as Diet 60, was made up as follows: extracted casein 18, sucrose 77.5, Salts 186 (9) 4.5, thiamine, riboflavin, and pyridoxine 10  $\gamma$  per gm of ration, calcium pantothenate 25  $\gamma$  per gm of ration, *D*-inositol 100  $\gamma$  per gm of ration, choline hydrochloride, 1 mg per gm of ration, and calciferol 0.25  $\gamma$  per gm of ration.

The extracted casein was prepared by refluxing 500 gm batches of commercial casein with 2 liters of 95 per cent alcohol, and filtering while hot on a Büchner funnel. This was repeated five times. The first reflux was carried out with 10 ml of concentrated sulfuric acid added to the alcohol.

Besides being low in vitamin A activity, Diet 60 is low in fat and all fat-soluble factors except vitamin D.

**Animals**—Black and white hooded rats were used in this work. Litters were obtained from the stock colony at an average weight of 35 gm and placed on the basal diet. A plateau in the weight curve began in 5 weeks at 90 to 130 gm. Within any one litter, the weights deviated by about  $\pm 7$  gm from the mean. Daily weights were taken until a plateau was assured, at which time the litter was started on experiment. Only one or two litters of six to eight animals each were started on depletion at a time. For the work reported here, litters were started at intervals over a 6 month period. Each litter was made into a complete and self-contained experiment, with usually only one rat on each supplemental variation. Thus, the final results represent the average of several small scale experiments. By the adoption of this procedure, assurance was given that the results obtained were not artifacts caused by variation between litters or other conditions of the experiment, such as deterioration of materials.

**Supplements**—Some workers (3, 4) have preferred to feed daily supplements by calibrated dropper. However, Sherman (1) has had success in dispersing the appropriate quantities of supplement solutions on a small quantity of basal ration, mixing, and feeding. He has shown conclusively that no loss of carotene occurs prior to consumption by the rat. This procedure was continued, but with some modifications. Since iron and copper salts are well known prooxidants, a special supplement diet has been used, identical with Diet 60, except that the salt mixture was omitted. Exactly  $1 \pm 0.1$  gm of this special diet was placed in the supplement cups. The carotene and antioxidant solutions were pipetted onto this and were allowed to disperse for 20 to 30 minutes. The fat or oil, if used, was then measured in and the contents of the jar mixed with a clean glass rod. 2 hours previously the regular feed jars had been removed from the cages. The supplement cups were offered to the rats, and the supplement was practically always consumed in less than 15 minutes. 2 hours later the

regular feed jars were replaced. By this procedure the possible complicity of inorganic salts in the destruction of carotene, either before or after consumption by the rat, was minimized. To compensate for the absence of salt mixture in this 1 gm. of feed, the salt content of the basal diet was increased from 4.0 to 4.5 per cent.

Crystalline carotene (Smaco, 90 per cent  $\beta$ -, 10 per cent  $\alpha$ -) was dissolved in Skellysolve B. The concentration of this solution was checked every 10 days. A new solution was made up every 6 weeks. Merck's synthetic  $\alpha$ -tocopherol was dissolved in Skellysolve B. Gossypol and dianilinogossypol were prepared as previously indicated (8). Gossypol was used in a peroxide-free ether solution in a concentration of 10 mg. per ml. Dianilinogossypol was used as the solution in reagent grade chloroform at a concentration of 2 mg. per ml.

Methyl inolate was prepared and stored as previously described (1). Its peroxide number was less than 2 milliequivalents per kilo at the end of the experiment. Swift's Silverleaf brand lard was used, and its peroxide number at the end of the experiment was less than 1.5 milliequivalents per kilo.

All rations and supplements were kept in a refrigerator room at all times. The gossypol was shown to be free of tocopherols by application of the Parker-McFarlane treatment and iron-bipyridine reagent as previously described (10).

### Results

The data in Fig. 1 show the average growth rates of male and female depleted rats receiving 2  $\gamma$  of carotene per day, alone, or with the addition of 0.1 gm. of lard, 0.1 gm. of hydrogenated coconut oil, 0.8 mg. of  $\alpha$ -tocopherol, or one of the fats plus the tocopherol.

Rats receiving carotene alone failed to grow appreciably, declined in weight, and died. Altogether, seventeen rats received this supplement with 100 per cent mortality by the end of 12 weeks. This differs from Sherman's results (1) in that his rats, receiving 2  $\gamma$  of carotene only, grew steadily throughout the experiment, although very slowly. This may be explained by the presence of the small amount of fat and antioxidant present in the dry yeast used in his basal diet. The initial xerophthalmia of the control rats, whose growth record is shown in Fig. 1, was cured in a week or two after the 2  $\gamma$  of carotene per day were received. However, the fat acid deficiency symptoms persisted and became progressively more acute. The skin around the eyes and nose became dry, caked, and scaly. The dry flakiness around the paws gradually changed to a moist appearing swollen dermatitis. There was dandruff on the coat which was rough, with many loose hairs.

Feeding of lard plus carotene to depleted rats had little beneficial effect on the growth response. Altogether, fourteen rats received this supplement and only two survived to 12 weeks. Fatty acid deficiency symptoms

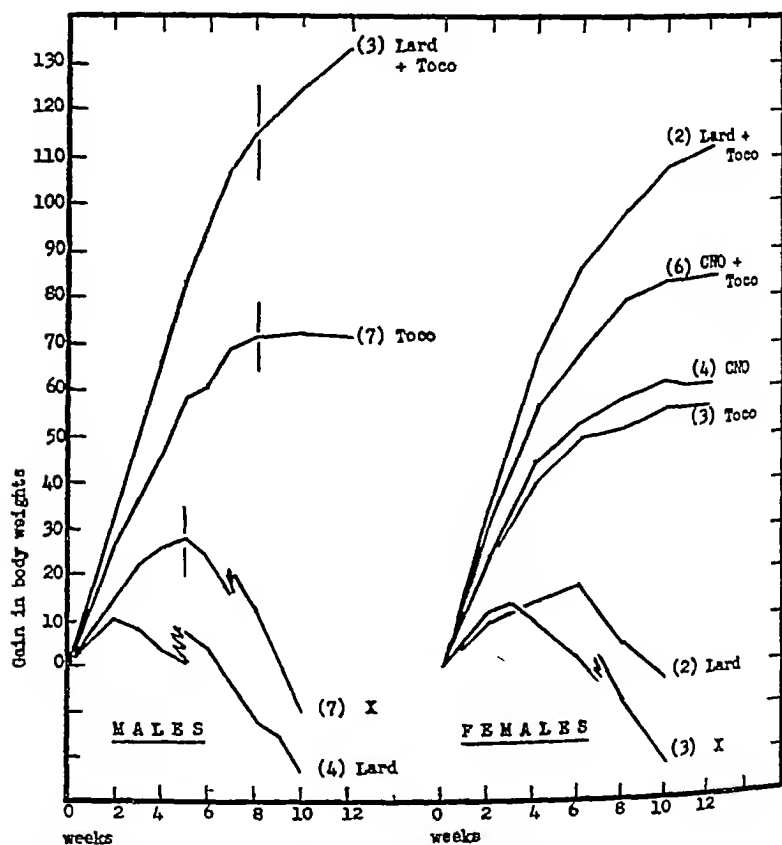


FIG 1 The growth responses of vitamin A deficient rats, whose weights reached a plateau, to daily supplements of 2 $\gamma$  of carotene (X), the carotene plus 0.1 gm of lard, the carotene plus 0.8 mg of  $\alpha$  tocopherol (TOCO), the carotene plus 0.1 gm of hydrogenated coconut oil (CNO), and the carotene with the indicated fats plus the tocopherol. The numbers in parentheses indicate the number of animals. Broken lines indicate one or more deaths.

were absent, but the rats developed a recurrence of xerophthalmia and a severe incoordination, with extreme leg weakness in those cases in which growth was evident. Thus, this lard appeared to have the same adverse effect as does pure methyl linolate, as shown by Sherman (1).

Feeding of carotene plus tocopherol (but no fat) resulted in some

growth, but an early plateau. No deaths occurred in this group. All vitamin A deficiency symptoms disappeared, but scaldiness around the paws and nose persisted. The feeding of hydrogenated coconut oil plus carotene resulted in growth about equal to that obtained on the tocopherol plus carotene supplement, with similar persistence of fatty acid deficiency symptoms. Since this coconut oil gave negative tests for tocopherol (10) or antioxidants (8), the possibility of a mechanical protection of carotene in its passage through the tract must be considered. Lard plus tocopherol plus carotene supported good growth. The coconut oil plus tocopherol and carotene resulted in better growth than was evident in the group receiving the coconut oil and carotene (with no tocopherol), but it was not as great as on lard plus carotene and tocopherol.

In a preliminary experiment to determine the effectiveness of gossypol as an internal antioxidant, Diet 60 was altered by including 10 per cent of lard and increasing the casein to 20 per cent. Rats were depleted on this ration and started on experiment. Of six rats receiving 16  $\gamma$  of carotene per day only, all were dead by the end of 3 weeks with weight losses ranging up to 36 gm. Six rats receiving 0.3 mg of pure gossypol plus the carotene showed an average weight gain of  $28 \pm 6$  gm in 4 weeks. Four rats receiving 0.3 mg of  $\alpha$ -tocopherol in addition to the carotene showed an average weight gain of  $44 \pm 12$  gm by the end of 4 weeks. This experiment looked promising and was repeated in greater detail.

The data in Fig. 2 show the average growth records of rats that received 1 mg daily doses of gossypol, dianilinogossypol,  $\alpha$ -tocopherol, or  $\alpha$ -tocopherol plus gossypol, in addition to 2  $\gamma$  of carotene and either 0.1 gm of lard or 25 mg of ethyl linolate. Diet 60 was used unaltered in this work.

From the results in Fig. 2, it is apparent that both gossypol and dianilinogossypol are quite effective internal antioxidants. Altogether, fifteen rats have received gossypol in addition to carotene plus a fatty material and satisfactory responses have been observed in each case. Ten rats have been on the dianilinogossypol supplement with good responses in all cases. The rats receiving linolate did not grow as well as those on the lard supplement. This may be due to the different quantities of these fatty materials used.

*Carotene Protection, in Vitro, and Inhibition of Rancidity Development by Cottonseed Products*—Crystalline carotene (Smaco, 90 per cent  $\beta$ -) was dissolved in pure ethyl oleate and was added to several commonly used feedstuffs in such quantities that the final mixtures contained 80  $\gamma$  of carotene per gm, and 30 per cent of ethyl oleate. The feedstuffs had been previously ground to pass a 24 mesh screen. The wheat products were fresh and all milled from the same batch of whole wheat.<sup>1</sup> The cottonseed

<sup>1</sup> The wheat milling fractions were obtained from the Pillsbury Flour Milling Company, through the courtesy of Mr. C. G. Harrel.

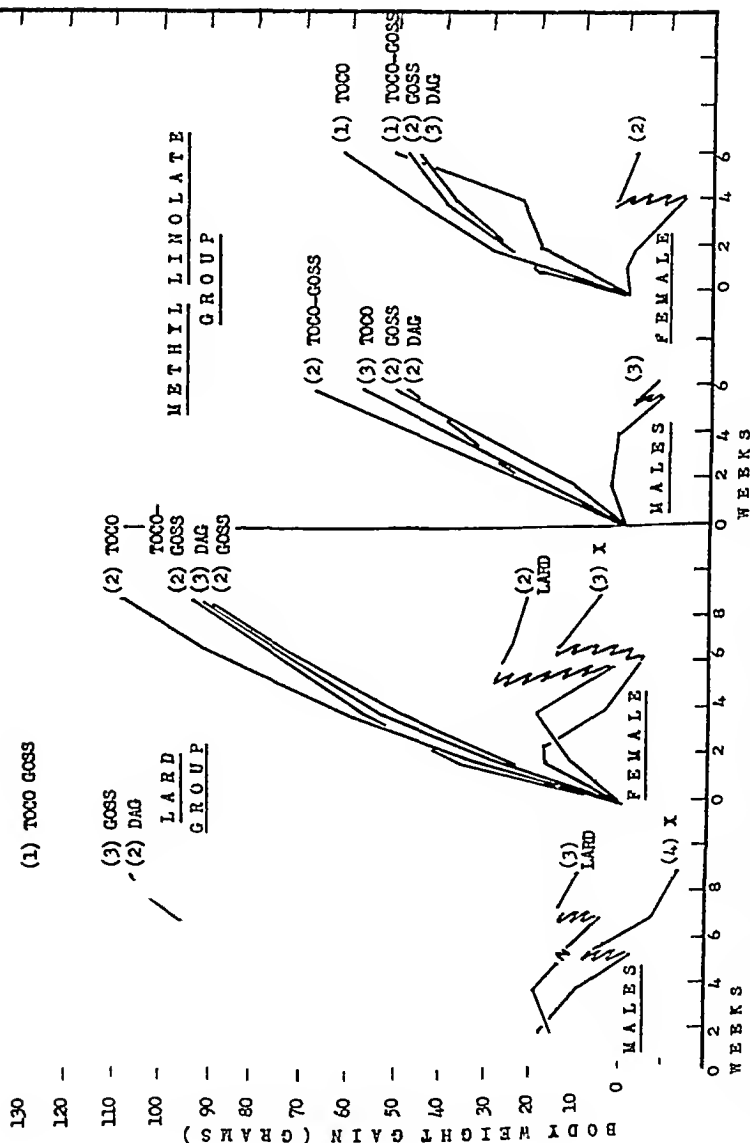


Fig. 2 The growth responses of vitamin A deficient rats whose weights reached a plateau to daily supplements of 2 g of carotene (X) to the carotene plus either 0.1 gm of lard or 25 mg of methyl linolate to the carotene with one of the fatty materials plus 1 mg of an antioxidant as indicated (TOCO) = tocopherol (GOSS) pure gossypol (DAG) pure diantlinogossypol. The numbers in parentheses indicate the number of animals. Broken lines in female one or more deaths.

oil meal and other products were obtained from the stock-feed barn of the Experiment Station. Carotene determinations were made by the method of the Association of Official Agricultural Chemists. Total tocopherol determinations were carried out on the 24 hour Skellysolve B extracts of the products by the iron-bipyridine method, as previously described (10). The samples were stored at 58° in open dishes. 2 gm aliquots were removed at 6, 20, and 31 days for carotene determinations. The percentage losses of carotene were calculated on the basis of the original determinations before storage.

TABLE I

*Stability of Carotene Added at a Level of 80  $\gamma$  per Gm to Feedstuffs Mixed with Ethyl Oleate at a 30 Per Cent Level and Stored Open at 58°*

Product	Original tocopherol content	Carotene loss in		
		6 days	20 days	31 days
	mg per gm	per cent	per cent	per cent
Wheat germ	0.28	16.5	33.0	35.0
Cottonseed oil meal	0.05	20.0	33.5	36.0
Red dog flour	0.08	25.5	43.0	53.7
Soy bean oil meal	0.07	34.0	54.5	58.3
Wheat bran	0.07	34.0	56.0	62.0
2nd clear flour	0.022	40.5	65.3	77.0
1st " "	0.016	50.5	77.0	86.5
Peanut oil meal	0.016	60.0	85.0	91.1
Patent flour, wheat	0.011	68.0	95.4	100
Whole wheat	0.03	71.0	90.5	98.0
Skim milk powder	0	97.2	100	
Corn starch	0	78.9	100	

The results are given in Table I. It is apparent that cottonseed oil meal provides good protection to carotene under these conditions, in spite of the fact that its tocopherol content is lower than several of the other products tested.

Some experiments were also carried out to determine the relative stability of cottonseed, soy bean, and peanut oil meals. The ground products were stored in half full jars at 60°, and the development of rancidity followed organoleptically. The results are shown in Table II. These can only be considered as rough estimates because of the type of experiment, but they do have practical implications. The age of the products studied varied from less than 2 months for peanut oil meal Sample III to more than a year, under practical conditions, for peanut oil meal Sample I and the cottonseed oil meal. The defatted ground cotton seeds were fresh. They had been extracted with Skellysolve until no determinable fat or tocopherols re-

maintained. When this product was added to peanut oil meals at a level of 10 per cent, the keeping quality of these meals was increased 4 to 5 times. This defatted, ground cotton seed contained 0.8 per cent free gossypol by isolation. The fat contents of the commercial oil meals used in this study varied from 4.8 to 5.7 per cent.

TABLE II  
*Organoleptic Keeping Times of Commercial Oil Meals*

Oil meals	Admixture at a 10 per cent level	Keeping time at 60 days
Cottonseed		70
Soy bean		56
Peanut, Sample I		14
" " II		28
" " "	Cottonseed oil meal	47
" " "	Soy bean oil meal	40
" " "	Ground, defatted cotton seeds*	107
" " III		38
" " "	Cottonseed oil meal	66
" " "	Soy bean oil meal	51
" " "	Ground, defatted cotton seeds*	202

\* Fresh, ground, cottonseed meals thoroughly extracted with Skellysolve B

#### DISCUSSION

The data show that pure gossypol and diamlinogossypol are about equally effective as carotene-preserving antioxidants when fed to rats, and are only slightly inferior to  $\alpha$ -tocopherol in this respect.

Defatted cotton seeds, containing gossypol, extended the keeping quality of peanut oil meals, *in vitro*, by 4 or 5 times, when mixed at a 10 per cent level into these meals. Commercial cottonseed oil meal proved to be equal to wheat germ in stabilizing a carotene solution in ethyl oleate. The tocopherol contents of these cottonseed products were much too low to explain their stabilizing action. Possibly this action is related solely to the gossypol or gossypol derivatives present.

Gossypol, or cottonseed products containing this factor, are known to be toxic to many animals. The minimum toxic dose for rats is said to be about 6 mg per day, orally (11). Other animals such as the hog or chicken are much more sensitive (12), while cattle or goats are, like the rat, quite resistant. Raw cotton seeds contain about 1 per cent of free gossypol. Commercial cottonseed oil meals contain from 0.05 to 0.20 per cent of free gossypol, with an average of about 0.10 per cent. Thus, the 1 mg per day level fed to rats in these experiments corresponds roughly to 10 per cent

cottonseed oil meal in the ration. This is a safe level to feed to most farm animals. In the pressing of cotton seeds, a fair amount of gossypol goes into the oil. This is eliminated during the alkali refining process.

Apparently, a large part of the toxicity of gossypol is related to its two carbonyl groups. Apogossypol, which is gossypol with the two carbonyl groups eliminated by strong alkali, is much less toxic than the original product (13). In dianilinogossypol, the free carbonyl groups of gossypol are tied up by the anilido complex. It has never been determined whether this compound has lost the high toxicity that is characteristic of the free gossypol. The data presented in this paper show that the dianilinogossypol has not lost the antioxidant activity characteristic of the free compound.

The demonstration of a carotene-preserving action of gossypol and cottonseed products in no way changes the situation relative to the toxic properties of these. However, it does suggest that a balance can be struck between the good and bad effects. A more efficient utilization and better preservation of carotene are especially significant during the present crisis in supplies of vitamin A-active materials for stock feeding. If the toxicity of gossypol can be lessened or destroyed while its antioxidant properties are maintained, a much more important and far reaching benefit can be derived from cottonseed products.

#### SUMMARY

1 Small amounts of lard fed with small doses of carotene to rats that were deficient in vitamin A and essential fat acids resulted in growth failure and death. This result was similar to the previously demonstrated effect of methyl linolate on carotene fed to such rats.

2 Pure gossypol and dianilinogossypol were effective antioxidants, at daily doses of 1 mg, for the preservation of carotene fed with lard or methyl linolate to depleted rats. These compounds were only slightly inferior to  $\alpha$ -tocopherol in this respect.

3 Cottonseed oil meal was equal to wheat germ in stabilizing a carotene solution in ethyl oleate, *in vitro*, and much superior in this respect to several other common feedstuffs. Defatted cotton seeds added to peanut oil meals at a 10 per cent level extended the keeping quality of the meals by 4 to 5 times.

4 The significance of these findings is discussed in relationship to commercial cottonseed products.

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# CONCERNING THE REINECKATE METHOD FOR THE DETERMINATION OF CHOLINE

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In the light of recent developments concerning the rôle of choline in nutrition and intermediary metabolism, the immediate need for a rapid quantitative chemical method for the determination of this substance is apparent. Most of the chemical methods employed to date have been based on the precipitation of choline as the reineckate, but other methods brought forth include the titrimetric determination of the trimethylamine formed by oxidative degradation of the choline (1-3), the precipitation of choline as the periodide with the subsequent liberation and titration of the iodine (4-7), and the determination of the mercury in the mercuric chloride double salt of choline (8, 9). An excellent recent review by Best and Lucas (10) includes a discussion of the estimation of choline.

Among the first to employ the reineckate method for the determination of choline were Kapfhammer and Bischoff (11) who dissolved the choline reineckate in acetone, evaporated the solvent, washed the residue with ether, and weighed it after drying. Greater sensitivity was obtained by Beattie (12) who, by subjecting the pink acetone solution of choline reineckate to colorimetry, could estimate around 0.3 mg. of choline in a concentration of 0.003 per cent with a maximum error of 3 per cent. Jacobi, Baumann, and Meek (13), Thornton and Broome (14), and Engel (15) applied photoelectric colorimetry to the acetone solution. The development of a brown color, by adding an iodine reagent to an aqueous solution of choline reineckate, was used as the basis of a method by Shaw (16). Later Marenzi and Cardini (17) described a colorimetric procedure in which the choline reineckate is oxidized by alkaline hydrogen peroxide and then treated with diphenylcarbazide in acid solution to yield a violet-red color (Cazeneuve's reaction for chromate). The authors claim this method will allow the estimation of as little as 15  $\gamma$  of choline.

For ordinary purposes, the method depending on photoelectric measurement of the color intensity of the acetone solution of the reineckate is as good as any in current use. Since this method has the advantages of simplicity and speed, the present communication will deal with an examination of the steps in the procedure and certain improvements that have been effected during the course of this investigation.

*Methods*

The details of the procedure finally adopted will be presented first, and a critical examination of the consecutive steps will then follow

The weighed sample, containing the equivalent of 2 to 5 mg of choline chloride, is placed in an alundum thimble of medium porosity (80 mm long  $\times$  22 mm in diameter) for extraction in a Soxhlet apparatus fitted with a 125 ml boiling flask. About 100 ml of methanol are used as the solvent and a boiling chip is added. The extraction is allowed to proceed for 24 hours on an electric hot-plate. With some finely divided materials such as flour, the tendency to form a hard cake makes it desirable to mix the sample intimately with No. 2 pulverized pumice to facilitate the extraction. The ratio of the sample to the pumice depends on the nature of the sample, convenient proportions for a few cereal products are given in Table I.

TABLE I  
*Proportion of Sample to Pumice for Extraction*

Sample	Sample weight	Pumice weight
	gm	gm
Flour	3	10
Bran	4	0
Wheat germ	2	0
" shorts	5	0
Ground wheat	5	7
" oats	2	7
" barley	3	7
" soy beans	1	8
" flax	3	7

The boiling flask containing the methanol extract is placed on a steam bath and, when only a few ml of solvent remain, 30 ml of a saturated solution of barium hydroxide are added and the heating is continued for 90 minutes. After the mixture is cooled, a drop of 1 per cent alcoholic thymolphthalein is added to the hydrolysate, and glacial acetic acid is introduced until the blue color is just discharged by 1 drop. The liquid is then filtered by suction through a sintered glass filter tube of medium porosity (15 to 30 ml capacity) into a 125 ml suction flask. The boiling flask is rinsed with small portions of distilled water and the rinsings are used to wash the filter, a total of about 15 ml of water being used. To the combined filtrate and washings, 6 ml of a 2 per cent solution of Remecke salt in methanol are added, and the flask is placed in a refrigerator at about 5° for 2 hours. The Remecke salt solution is freshly prepared each week, and it is stored in a refrigerator.

The choline reneckate precipitate is separated by filtering with suction

through a 30 ml sintered glass filter tube of medium porosity into a 500 ml suction flask. When the precipitate is dry, it is washed three times with 25 ml portions of *n*-propanol, and again dried by means of the suction. A tube graduated at 10 ml (14.5 cm long  $\times$  1 cm in diameter) is placed inside of the suction flask so that the stem of the filter tube can be inserted into its mouth. The choline reineckate on the filter is dissolved in a few ml of acetone and, by means of gentle suction, the solution is drawn into the 10 ml tube. Small additions of acetone are used to wash the filter until the volume of filtrate is 10 ml. After the pink solution is mixed, a few ml are transferred to a cuvette or tube for photoelectric determination, at 526 m $\mu$ , of the choline reineckate concentration. A color standard

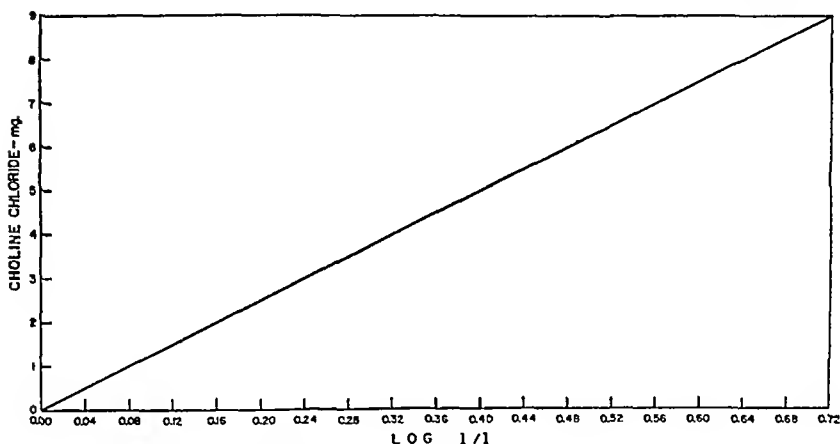


FIG 1 Color intensities obtained with known quantities of pure choline chloride

of methyl red (40 ml of 6.25 mg per cent aqueous methyl red solution + 460 ml of 0.1 M citrate buffer, pH 3.7) is used to set the instrument before each measurement. The 10 ml of acetone solution are more than sufficient for measurements with the Beckman quartz spectrophotometer used in the present study. In other instruments cuvettes or tubes requiring larger volumes may be employed, and in these cases the acetone solution must be diluted accordingly.

The final result, expressed in mg of choline chloride, is obtained by reference to a calibration curve, Fig 1, constructed from the color intensities ( $\log I_0/I$ ) given by solutions of known choline chloride concentration.

### Results and Discussion

*Extraction of Choline and Its Compounds from Natural Sources*—A variety of solvents has been used for the extraction of choline-containing lipids

from various sources, but methanol extracts have been found by Engel (15) to yield the highest values for choline by the reineckate method, and the advantages of using methanol have been corroborated by Rhian *et al* (18). In the present work it was found to be of greater advantage to employ Soxhlet extractors with standard taper parts rather than the Bailey-Walker apparatus as used by Engel, chiefly because a single lot of 100 ml of methanol could be used instead of the three separate 30 ml lots, and the subsequent evaporation of the solvent and hydrolysis of the residue could be carried out conveniently in the same Soxhlet boiling flask without transfer.

The time required for complete extraction depends, of course, on the state of subdivision of the sample. It was observed that with some finely divided materials, such as flour, the tendency to form a hard cake may increase the necessary extraction time to 72 hours. However, com-

TABLE II

*Effect of Use of Pumice on Rate of Methanol Extraction of Whole Wheat Flour in Soxhlet Apparatus*

	Choline in extract (log $I_0/I$ ) after extraction for			
	24 hrs	48 hrs	68 hrs	72 hrs
Without pumice	0.190	0.233		0.260
With pumice	0.258	0.266	0.263	0.263

plete extraction was found to be effected for most materials in 24 hours by the inclusion of pumice, as shown by the example in Table II.

*Liberation of Choline by Hydrolysis*—The use of barium hydroxide for the hydrolysis was established by Erickson *et al* (6), and the procedure employed in the present case is essentially that of Jacobi *et al* (13). It was found unnecessary to utilize Engel's more cautious technique (15) of applying reduced pressure with bath temperatures lower than 100° for removal of methanol prior to hydrolysis. In the case of wheat germ extract, the hydrolysis was virtually complete after 45 minutes on the steam bath, but a 90 minute period was used for cereal products to allow an adequate margin of safety. Thymolphthalein is to be recommended instead of phenolphthalein for the neutralization, since the color does not fade and the color change is easier to detect.

The specificity of the method depends largely on the nature of the hydrolysis and subsequent neutralization. This is demonstrated by the data in Table III. Separate aliquots from a given extract were subjected to hydrolysis by 10 per cent potassium hydroxide and saturated barium hydroxide, equal volumes of hydrolysate were neutralized with glacial

acetic acid to pH 8 to 9 in one case, and with concentrated hydrochloric acid to pH 2 to 3 in the other. The color intensities of the acetone solutions of the reneekates obtained are given in Table III. The alkaline hydrolysis under the conditions given does not destroy the choline. This has been proved by Engel's (15) recovery experiments, and confirmed in the present work (Table III).

Compounds, other than choline, that form insoluble reneekates exist in many biological materials or can be liberated from them on hydrolysis. If this difficulty could not be overcome, the reneekate method would be of

TABLE III

*Effect of Nature of Alkali Used for Hydrolysis and pH of Precipitation on Intensity of Reneekate Color*

The values are given in terms of  $\log I_0/I$

Source of methanol extract	pH of pptn	Hydrolysis with 10 per cent KOH	Hydrolysis with saturated Ba(OH) <sub>2</sub>
Wheat germ	8-9*	0.23	0.21
	2-3†	0.55	0.37
Soy bean	8-9	0.51	0.51
	2-3	0.54	0.45
Yeast	8-9	0.50	0.46
	2-3	0.67	0.69
Calf liver	8-9	0.75	0.72
	2-3	0.75	0.63
Hog brain	8-9	0.32	0.31
	2-3	0.34	0.33
Lecithin	8-9	0.53	0.53
	2-3	0.53	0.56
Choline chloride	8-9	0.15‡	0.14
	2-3	0.15	0.15

\* CH<sub>3</sub>COOH was used for neutralization to thymolphthalein

† HCl was used for neutralization to Congo red

‡ 0.15 with no hydrolysis

much less value, but the problem can be circumvented to a considerable degree by precipitating the reneekate in an alkaline medium. The reneekates of compounds with carboxyl groups, such as betaine or carnitine, are soluble in alkali, as shown by Strack and Schwaneberg (19), and hence they may be separated from choline reneekate when the precipitation occurs at an elevated pH. The higher values that accompany the neutralization to the acid pH (Table III) are probably due to the presence of betaine or similar reneekates. Interference from still other non-choline compounds capable of forming reneekates appears to be eliminated when barium hydroxide is employed for hydrolysis, since the values obtained

were, in some cases, less than the corresponding values found when potassium hydroxide was used. This would indicate that the barium effected removal of the interfering substances, presumably by precipitating them as compounds which would be removed by the filtration preceding the addition of Reinecke salt.

When hydrolysis with barium hydroxide was carried out on both soy bean and calf liver extracts, smaller values were obtained from the hydrolysates neutralized to the lower pH. This point was checked repeatedly, and the results were consistent, however, no ready explanation for this finding is apparent.

No significant difference in result was obtained by the purification of neutralized barium hydroxide hydrolysates of wheat germ extracts either by extraction with ether or by adsorption of the choline on permutit and elution with salt solution according to Horowitz and Beadle (20).

*Precipitation and Isolation of Choline Reineckate*—Most investigators in the past have added an equal volume of a saturated aqueous solution of ammonium reineckate to a choline solution for the precipitation of choline reineckate. However, Jacobi *et al* (13) employed 5 ml of a 2 per cent solution of the Reinecke salt in methanol for precipitation of the combined filtrate and washings of the hydrolysate, and Engel (15), following this procedure, used 6 ml of the reagent. After the use of both the aqueous and alcoholic solutions of Reinecke salt were compared during the present investigation, it was observed that the latter gave more consistent and reliable results.

The time allowed for complete precipitation is another factor that has varied considerably between laboratories. Shaw (16) did not let the reaction mixture stand at all, Thornton and Broome (14) allowed 10 minutes at room temperature for the reaction, Beattie (12) and Marenzi and Cardini (17) 20 minutes at ice bath temperatures, Jacobi *et al* (13) 12 hours in the cold, and Engel (15) 4 hours at 3°. All except the last two used a saturated aqueous Reinecke salt reagent. The effects of precipitation time and temperature on the intensity of the color of acetone solutions of choline reineckate, prepared from the same hydrolysate of wheat germ extract, are shown in Table IV. It is clear that the reaction is more complete at the lower temperature, and also that 2 hours can be considered a precipitation period with a wide margin of safety. Similar results were obtained when pure choline solutions were substituted for hydrolysate.

Ethanol has been commonly used to wash the choline reineckate free of the Reinecke salt reagent, but the solubility of the precipitate in ethanol is appreciable, as shown by Engel (15) who therefore employed ethanol cooled to 3°. The difficulty of keeping the ethanol that cold during the washing and the danger attending a rise in temperature led to a search for

another washing liquid that could be used at room temperature In Table V the qualitative solubilities of both Reinecke salt and choline reineckate are indicated Those liquids in which choline reineckate was insoluble (no color) were tested as solvents for Reinecke salt in the hope of finding one which would dissolve this reagent Propyl, butyl, and amyl alcohols all fell into this group, but *n*-propanol was selected as the best because

TABLE IV

*Effects of Time and Temperature on Precipitation of Choline Reineckate*

6 ml of 2 per cent Reinecke salt in methyl alcohol were added to approximately 30 ml of hydrolysate

Temperature	Log I <sub>0</sub> /I after			
	0 5 hr	1 0 hr	1 5 hrs	2 0 hrs
C				
5	0 188	0 207		0 210
25	0 148		0 158	0 155

TABLE V

*Relative Solubilities of Choline Reineckate and Reinecke Salt in Various Media at 30°*

Solvent	Relative intensity of pink color of filtered solutions	
	Choline reineckate	Reinecke salt
Methanol	+++	
Ethanol	+++	
<i>n</i> -Propanol	—	++
Isopropanol	Trace	+
<i>n</i> -Butanol	"	+++
Isobutanol	+	
Isoamyl alcohol	Trace	+++
Hexone	++	
1,4-Dioxane	++	
<i>n</i> -Butyl ether	—	—
Isobutyl ether	—	—
Carbon tetrachloride	—	—
Benzene	—	—
Cellosolve	+++	

of its lower boiling point and viscosity compared with the higher alcohols Isopropanol was discarded, since Reinecke salt was less soluble in it

*Light Absorption Characteristics of Reineckates*—A maximum absorption in the range of 500 to 550  $m\mu$  was reported for the acetone solution of choline reineckate by Thornton and Broome (14), Jacobi *et al* (13) found the maximum to be between 520 and 530  $m\mu$ , and Marenzi and Cardini (17) ob-



served greatest absorption with a  $530\text{ m}\mu$  filter when 60 per cent acetone was used as the solvent. In all of these cases, filters were employed to estimate maximum absorption.

In order to obtain a more complete and accurate picture of the absorption characteristics, not only of choline reineckate, but also of the Reinecke salt reagent and of acetylcholine reineckate, measurements were made over the range 360 to  $1000\text{ m}\mu$  with the Beckman quartz spectrophotometer and corex cells of 1.000 to 1.002 cm width. The two reineckates were prepared by the addition of an alcoholic solution of Reinecke salt to the

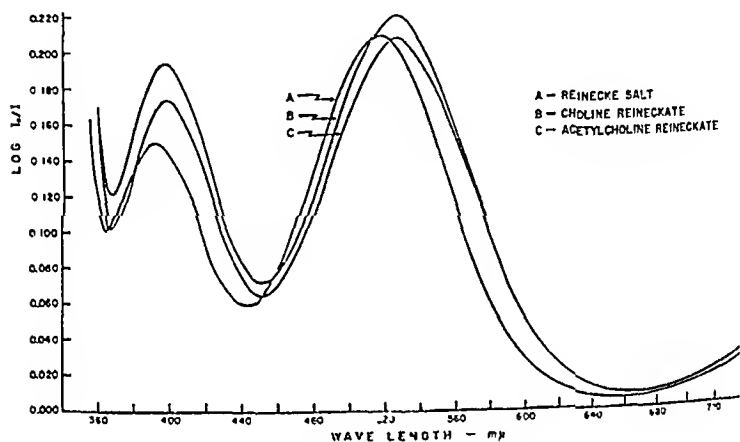


FIG 2 Absorption curves for acetone solutions of 0.002 M concentration. 1 cm cells were used.

respective aqueous solutions of choline chloride and acetylcholine chloride. The recrystallized compounds gave the following analyses:

$\text{NH}_4[\text{Cr}(\text{SCN})_4(\text{NH}_3)_2] \cdot \text{H}_2\text{O}$	Theory, N 27.66, found, 27.90
$[\text{Cr}(\text{SCN})_4(\text{NH}_3)_2]\text{N}(\text{CH}_2)_3\text{CH}_2\text{CH}_2\text{OH}$	" " 23.20, " 23.26
$[\text{Cr}(\text{SCN})_4(\text{NH}_3)_2]\text{N}(\text{CH}_2)_3\text{CH}_2\text{CH}_2\text{OCOCH}_3$	" " 21.11, " 21.35

Pure acetone was used as the solvent in the absorption measurements, and the cells were covered with glass lids to prevent evaporation.

The absorption curves are given in Fig 2 and the maximum for choline reineckate at  $526\text{ m}\mu$  was used in the present method. From 740 to  $1000\text{ m}\mu$  the values for  $\log I_0/I$  rose gradually and evenly in all three cases. The specific extinction coefficient for choline reineckate at  $526\text{ m}\mu$  calculated for 1 mg of choline chloride, 1 cm thickness, and a volume of 10 ml, is 0.080 (Fig 1). The molecular extinctions of the reineckates ( $1/\text{cd log}$

$I_0/I$  may be obtained from Fig 2 where  $c = 0.002$  M, and  $d = 1$  cm. Thus, choline reineckate at  $526\text{ m}\mu$  has a molecular extinction of  $0.222 - 1 \times 0.002 = 111.0$ , acetylcholine reineckate at  $526\text{ m}\mu$  has a value of  $104.5$ , and Reinecke salt at its maximum of  $517\text{ m}\mu$  equals  $105.5$

## SUMMARY

Each step in the reineckate method for the determination of choline was examined in detail. The procedure finally adopted included simplifications and improvements in certain of the steps.

The light absorption characteristics of acetone solutions of Reinecke salt, choline reineckate, and acetylcholine reineckate were determined over the range  $360$  to  $1000\text{ m}\mu$ .

The author wishes to express his appreciation to Dr. Betty Sullivan and Miss M. A. Howe for helpful criticisms and suggestions.

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# THE DETERMINATION OF TOCOPHEROLS WITH IRON-BIPYRIDINE REAGENT IN THE PRESENCE OF FATS\*

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Emmerie and Engel (1) were the first to use the iron- $\alpha, \alpha'$ -bipyridine reaction for the determination of tocopherols. It was assumed by these authors and others (2-7) that fat interfered with this reaction. In order to overcome these difficulties, elaborate procedures were used to separate the tocopherol from the fat. These procedures, especially when carried out on biological material, very frequently entail considerable losses of tocopherol. Therefore a more reproducible method for the determination of tocopherol in the presence of fats would be desirable.

The following experiments deal with the influence of fats on the tocopherol reaction with iron- $\alpha, \alpha'$ -bipyridine.

## Method

The apparatus used for the photometric measurements was a Coleman universal spectrophotometer. All determinations were performed at the wave-length of 515 m $\mu$ , with the reagent used by Devlin and Mattill (6). This reagent, as recommended by the laboratories of Merck and Company to Devlin and Mattill, contains 250 mg of FeCl<sub>3</sub> and 500 mg of  $\alpha, \alpha'$ -bipyridine in 1000 cc of glacial acetic acid. To 2 cc of petroleum ether<sup>1</sup> containing the  $\alpha$ -tocopherol<sup>2</sup> and oils 10 cc of the reagent were added and the development of the color recorded approximately at minute intervals within an accuracy of 3 seconds in the timing and of 1 per cent or less in the extinction for readings of 0.1 to 0.5 when a 1.0 cm square cell was used.

The tests were generally carried out in the following way. Petroleum ether solutions containing 10 per cent of the respective fats and oils and a second set of petroleum ether solutions containing from 25 to 200  $\gamma$  of  $\alpha$ -tocopherol per cc were prepared. 2 cc of petroleum ether were pipetted

\* Aided by a grant from the John and Mary R. Markle Foundation.

<sup>1</sup> Skellysolve B, purified by shaking with concentrated sulfuric acid and washing with water, dilute sodium hydroxide, and five times with water, dried over anhydrous sodium sulfate and distilled.

<sup>2</sup> We are indebted to Dr. R. D. Shaner of Hoffmann-La Roche, Inc., for supplying us with  $\alpha$ -tocopherol.

into the comparison cell of the spectrophotometer, followed by 10 cc of iron-bipyridine reagent. Into an identical cell were pipetted 1 cc of the oil solution and 1 cc of petroleum ether containing from 0 to 200  $\gamma$  of  $\alpha$ -tocopherol, again followed by 10 cc of reagent. At the instant when the reagent was added, the stop-watch was started and the color recorded for 10 minutes.

#### EXPERIMENTAL

The effect of acids and alkali upon the quantitative recovery of pure tocopherol has been repeatedly studied (8, 3). Under selected conditions, 90 to 100 per cent recoveries have been obtained. When these experiments were repeated by the same procedures in the presence of fats, such as sesame oil or lard, the extinctions found with the above iron-bipyridine reagent indicated recoveries of from 20 to 70 per cent. These decreases were attributed originally to the possible destructive effect of soaps and fatty acids upon the tocopherol, but it was found later that the addition of the fat to  $\alpha$ -tocopherol solutions without further procedures, namely without hydrolysis, always lowered the extinction of the iron-bipyridine color. This result demonstrates that the decrease of the extinction can be brought about also without destruction of tocopherol.

Curve I in Fig. 1 shows the standard curve given by amounts of from 0 to 200  $\gamma$  of pure  $\alpha$ -tocopherol, it indicates that the Beer-Lambert law is followed up to an extinction of approximately 0.4, which corresponds to about 100  $\gamma$  of  $\alpha$ -tocopherol. All readings were taken exactly 10 minutes after the reagent had been added.

The other curves in Fig. 1 were obtained by using 1.9 cc of petroleum ether containing the various amounts of  $\alpha$ -tocopherol and 0.1 cc of the respective fats and oils. Maximum readings within the first 10 minutes after addition of the reagent were plotted.

Curve II of Fig. 1, with sesame oil, is typical of the effect of fats on the slope of the curve. One sees that it requires 200  $\gamma$  of tocopherol to produce the extinction given by 33  $\gamma$  in a pure  $\alpha$ -tocopherol solution. With another sample of sesame oil these values checked within 5 per cent. These curves passed through the zero<sup>3</sup> point, because there was no measurable concentration of substances present capable of reducing ferric ion. This is further illustrated by Curves III, IV, and V in Fig. 1. It is important to note that these five curves have different slopes, and it is from these slopes that one can see the varied effect of oils and fats on the color produced by the same amount of tocopherol.

<sup>3</sup> For the oils referred to in Fig. 1, an extinction of from 0.001 to 0.003 was obtained. It is assumed that this was due to the presence of tocopherol or other impurities in such small amounts as to be without influence on the determination of a measurable quantity of  $\alpha$ -tocopherol.

The fat used for plotting Curve V of Fig 1 was obtained from a rat of 300 gm weight, which had been kept throughout life on a vitamin E-deficient diet After removal of the skin and the intestines, the whole animal was shredded in a Waring blender and extracted for 4 days with methyl alcohol and petroleum ether The combined extracts were evaporated, the residue was exposed to 2 N methyl alcoholic potassium hydroxide for 10 minutes at 70°, and the remaining fat was recovered by repeated shaking with petroleum ether The latter was washed and evaporated and the residue taken up in benzene and filtered through a 12 × 50 mm column of strongly adsorbing floridin<sup>4</sup> earth After evaporation of the benzene, a nearly colorless oil resulted, which was practically free from tocopherols

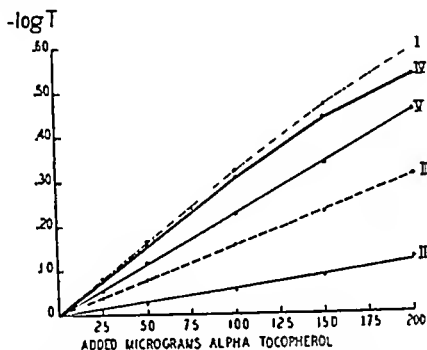


Fig 1 Curve I, 2 cc of Skellysolve containing amounts of  $\alpha$ -tocopherol as indicated on the abscissa plus 10 cc of iron-bipyridine reagent Curve II, 1.9 cc of Skellysolve and 0.1 cc of sesame oil containing amounts of  $\alpha$ -tocopherol as indicated on the abscissa plus 10 cc of iron-bipyridine reagent Curve III, same as for Curve II with coconut oil replacing the sesame oil Curve IV, same as for Curve II with lard replacing the sesame oil Curve V, same as for Curve II with rat fat replacing the sesame oil

and carotenes and which was used for the experiment 0.1 cc of this oil reduces the iron-bipyridine color by about 30 per cent, as seen in Curve V of Fig 1, a fact which is of significance for the determination of tocopherol in animal tissue

The method used for the determination of tocopherol in the presence of oils is demonstrated in Fig 2 which records the results from a sample of sesame oil to which 1.38 mg of  $\alpha$ -tocopherol per cc had been added Since 0.2 cc of this oil was used for the tests, the solution contained 278  $\gamma$  of  $\alpha$ -tocopherol To this original solution, further additions of  $\alpha$ -tocopherol were made (Fig 2) From the original reading and the slope of the curve, it is possible to calculate the original concentration of tocopherol from the

<sup>4</sup> Courtesy of the Floridin Company, Warren, Pennsylvania

similar triangles  $ABO$  and  $ACF$  with the extinction  $\epsilon_1(OB)$  for the unknown ( $x$ ) and  $\epsilon_2(CF)$  for  $x + a$  micrograms of  $\alpha$ -tocopherol  $x$  as the only unknown can be calculated from the following equation

$$\epsilon_1 x = \epsilon (x + a)$$

This calculation indicated the presence of 1.40 mg of  $\alpha$ -tocopherol per cc as compared to the 1.38 mg per cc which had been added to the oil. In an experiment with rat fat treated as the one used for plotting Curve V in Fig. 1, 240 mg of the fat and 330  $\gamma$  of  $\alpha$ -tocopherol were diluted in 5 cc of petroleum ether. 1 cc of the mixture with the standard reagent gave an extinction of 0.101, further addition of 110  $\gamma$  of  $\alpha$ -tocopherol raised the extinction to 0.268. From these two extinctions a value of 66.5  $\gamma$  of  $\alpha$ -tocopherol per cc of the petroleum ether mixture can be calculated as compared to the 66  $\gamma$  which had been added. Extrapolation was carried out in a similar way by Hodson and Norris (9) for the determination of riboflavin.

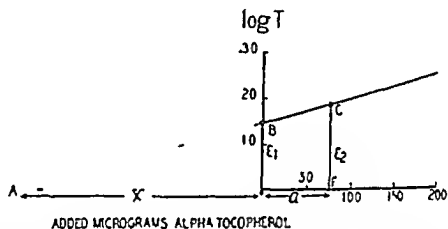


FIG. 2 Sesame oil with added tocopherol graphically presenting the extrapolation method.

The above procedure is necessary for two reasons. First, different fats produce curves with different slopes, as shown in Figs. 1 and 4, and secondly, the concentration of fat also affects the extinction, as shown by the curves in Fig. 3. These latter curves were obtained by adding the same amount of tocopherol to increasing fat concentrations in petroleum ether. The first additions of fat produce the greatest percentage effect on the extinctions. In the sesame oil curve, the addition of 5 mg of the oil lowers the extinction from 0.675 to 0.55, with approximately similar results for wheat germ oil. In Curve III of Fig. 3, the influence of the addition of 112  $\gamma$  of  $\alpha$ -tocopherol to varying amounts of rat fat was studied. The rat fat was the same as had been used for the experiment described in Curve V of Fig. 1. The result resembles those obtained with sesame oil and with wheat germ oil.

The fact that only the nature and the concentration of the fats and oils and not the tocopherol-fat ratio influences the results in the range selected for the determination is clearly indicated in Figs. 1 and 4. Even though in the experiments recorded in Figs. 1 and 4 the tocopherol-fat ratio is changed from 100.0025 to 100.02, the resulting curves are linear, and there-

fore justify the use of the extrapolation method without correction for the change in the tocopherol-fat ratio. The fact that the curves in Fig 3 are not linear is therefore not due to the change in the tocopherol-fat ratio but due to the absolute changes of the fat concentration. It is to be noted that from each individual point in the curves of Fig 3 a linear curve similar to those in Figs 1 and 4 could be obtained by different additions of tocopherol when the fat concentration was kept constant.

From the three curves in Fig 3 it becomes evident that for quantitative tocopherol determinations it is necessary either to eliminate fats com

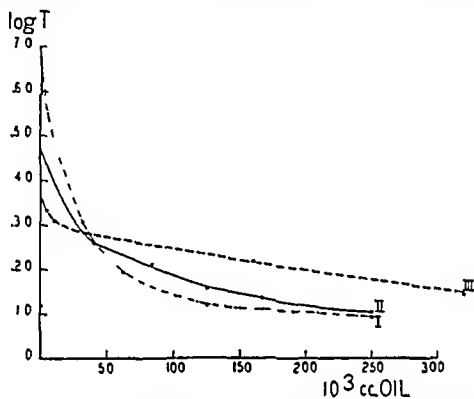


FIG 3 Curve I, amounts of sesame oil as indicated on the abscissa made to 2 cc with Skellysolve containing 250  $\gamma$  of  $\alpha$ -tocopherol plus 10 cc of iron-bipyridine reagent. Curve II, amounts of wheat germ oil as indicated on the abscissa made to 2 cc with Skellysolve containing 150  $\gamma$  of  $\alpha$ -tocopherol plus 10 cc of iron-bipyridine reagent. Curve III, amounts of rat fat as indicated on the abscissa made to 2 cc with Skellysolve containing 112  $\gamma$  of  $\alpha$ -tocopherol plus 10 cc of iron-bipyridine reagent.

pletely, with the great risk of losing considerable amounts of tocopherol, or to use the method described above. The depressing effect of wheat germ oil in the determination of tocopherol was observed by Parker and McFarlane (3) who state that "the more dilute the oil solution, the higher was its apparent tocopherol content." Also, Emmerie and Engel (1) noted that the color of ferrous bipyridine was sometimes obscured by substances present in oil. All these workers and others tried to overcome these effects by hydrolysis of the fats.

To demonstrate further the complexities of this effect, the curves obtained by adding known amounts of tocopherol to various fats are shown in Fig 4. As indicated, 0.05 or 0.1 cc of the particular fat was added to 1.95 or 1.90 cc of petroleum ether containing varying amounts of tocopherol. As before, 10 cc of the iron-bipyridine reagent were used, and





# STUDIES OF THE MECHANISM OF THE REACTION OF TOCOPHEROL WITH IRON- $\alpha, \alpha'$ -BIPYRIDINE IN THE PRESENCE OF FATS I\*

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In previous papers (1, 2), experiments were described for the quantitative determination of  $\alpha$ -tocopherol in the presence of fats and oils. With the better method used (2), it was found that the amount of ferrous bipyridine color produced by a fixed weight of tocopherol<sup>1</sup> was depressed in the presence of fats, the extent of depression depending upon the nature and concentration of the particular fat or oil. In this paper, experiments are presented which have yielded some information on the cause of this effect.

In the majority of these experiments, 10 cc of the iron-bipyridine reagent were added to 2 cc of purified Skellysolve B containing the tocopherol or other substances. The reagent contained 250 mg of  $\text{FeCl}_3$  and 500 mg of  $\alpha, \alpha'$ -bipyridine per 1000 cc of glacial acetic acid. The color development was studied in a Coleman universal spectrophotometer, with a 1 cm square cell at a wave-length of 515  $\text{m}\mu$ . All determinations were carried out at room temperature, since it was found that the extinction of the fat-free and fat-containing solutions at 37° was the same as at 20°.

Since upon exposure of fat-free and fat-containing solutions to light from three 200 watt bulbs at 50 cm the maximum decrease in the extinction was approximately 1 per cent in 15 minutes for solutions of different extinctions, all solutions were kept either in diffuse daylight or in darkness.

*Influence of Glycerol, Oleic Acid, Ethyl Alcohol, and Acetone*—The addition of glycerol or oleic acid in concentrations from 2 to 100 mg had, if it had any, a slightly elevating effect upon the color development.

Substitution of the glacial acetic acid in the reagent by 100 per cent ethyl alcohol did not nullify the color-depressing effect of fats. In a typical experiment in which alcohol was substituted for glacial acetic acid, the extinction of 100  $\gamma$  of  $\alpha$ -tocopherol was depressed from 0.125 to 0.056 when 100 mg of a particular sesame oil sample were added. With glacial acetic acid, the respective values were 0.42 and 0.06. These results demonstrate

\* Aided by a grant from the John and Mary R. Markle Foundation.

<sup>1</sup> We are indebted to Dr. R. D. Shaner of Hoffmann-La Roche, Inc., for supplying us with  $\alpha$ -tocopherol.

with an old sample of sesame oil and olive oil when ferrous acetate, obtained by dissolving  $\text{FeCO}_3$  in glacial acetic acid, was used in place of the  $\text{FeCl}_3$ . All these experiments prove that it is not the tocopherol but the ferrous ion which interacts with the fat.

These results suggest that ferrous ion and fat may form some sort of a complex, the stability of which is of the same order of magnitude as that of the ferrous bipyridine complex, or that peroxides present in the fat bleach the ferrous bipyridine complex. Curves II and III in Figs 3 and 8 are typical for all natural fats examined, the magnitude of the depression of the color, as well as the slope of the curves, varies with the nature and concentration of the fats. It must be emphasized that the absorption curves of the samples used in Curves I and III of Fig 3 were practically identical, which indicates that the ferrous bipyridine compound producing the color in the presence of fats is the same as in the absence.

That the color depression is probably not due to the presence of peroxides is demonstrated in Fig 4 (Curves I to IV). In Curve II of Fig 4, the results of an experiment are recorded in which sesame oil dissolved in Skellysolve was shaken with hydroquinone and  $\text{Na}_2\text{SO}_3$  in the presence of methanol for several hours. Curves I and II in Fig 4 should be identical if peroxides were the cause of the color depression, because peroxides were removed from the fat by the treatment with hydroquinone and  $\text{Na}_2\text{SO}_3$ . It is to be noted that the two solutions used for the experiment differed only in that the solution for Curve II contained 100 mg of the treated sesame oil.

Further evidence against the effect being caused by peroxides is presented in Curves III and IV of Fig 4. In this experiment, 28 mg of hydrogenated, peroxide-free sesame oil<sup>3</sup> were used. In view of the poor solubility of the hydrogenated sesame oil in Skellysolve, benzene was substituted for the Skellysolve after separate experiments had shown no difference in the color obtained with the two solvents.

A further argument against peroxides is the fact that the color increases with time in the presence of fats, in some instances (Curve II in Fig 2) it reaches the same maximum as in the absence of fats. If peroxides had oxidized the ferrous ion, the gradual formation of the ferrous bipyridine complex would be impossible.

Moreover, it is well known, that ferric ion catalyzes the decomposition of peroxides. Therefore experiments were carried out in which tocopherol and bipyridine were added to fat-ferric mixtures immediately and after 16 hours standing without essential difference in the type of curves ob-

<sup>3</sup> We are indebted to Dr D. Rittenberg for the preparation of the hydrogenated sesame oil.

tained Since the exposure of fat to ferric ion should destroy the peroxides, the conclusion seems justified that the color depression is not due to the presence of peroxides in the fat

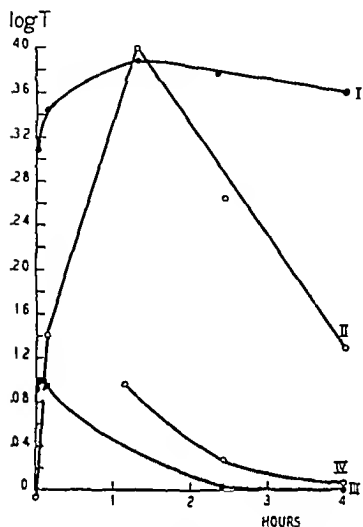


FIG 3

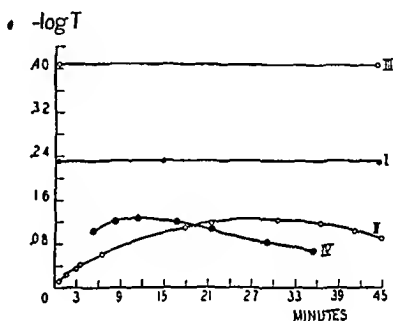


FIG 4

FIG 3 Curve I, 100  $\gamma$  of  $\alpha$ -tocopherol dissolved in 2 cc of Skellysolve plus 10 cc of glacial acetic acid containing 2.5 mg of  $\text{FeCl}_3$  and 5 mg of  $\alpha, \alpha'$ -bipyridine Curve II, 2 cc of Skellysolve containing 100  $\gamma$  of  $\alpha$ -tocopherol and 90 mg of sesame oil plus 5 cc of glacial acetic acid containing 2.5 mg of  $\text{FeCl}_3$ , followed after 30 seconds by 5 cc of glacial acetic acid containing 5 mg of  $\alpha, \alpha'$ -bipyridine Curve III, 2 cc of Skellysolve containing 100  $\gamma$  of  $\alpha$ -tocopherol and 90 mg of sesame oil plus 10 cc of glacial acetic acid containing 2.5 mg of  $\text{FeCl}_3$  and 5 mg of  $\alpha, \alpha'$ -bipyridine Curve IV, the same as for Curve III except that sesame oil was replaced by 90 mg of olive oil

FIG 4 Curve I, 2 cc of Skellysolve plus 5 cc of glacial acetic acid containing 12  $\gamma$  of ferrous ion, followed after 30 seconds by 5 cc of glacial acetic acid containing 5 mg of  $\alpha, \alpha'$ -bipyridine Curve II, same as for Curve I except that the Skellysolve contained 100 mg of sesame oil which, after having been dissolved in Skellysolve, was treated by shaking for several hours with hydroquinone and  $\text{Na}_2\text{SO}_3$  in the presence of methanol, the latter was carefully washed out with water Curve III, 2 cc of benzene plus 3 cc of glacial acetic acid containing 15  $\gamma$  of ferrous ion, followed after 30 seconds by 3 cc of glacial acetic acid containing 3 mg of  $\alpha, \alpha'$ -bipyridine Curve IV, same as for Curve III except that the benzene contained 28 mg of hydrogenated peroxide-free sesame oil The sesame oil was dissolved in Skellysolve for the hydrogenation, after it was chilled, the hydrogenated oil precipitated and was dissolved in benzene Blank and test solutions used for Curves III and IV were kept in a water bath of 35–45° in the intervals between readings

**Carotenes**—Results obtained by means of the reducing effect of carotenes also show color depression in the presence of fat (Curves IV and V in Fig 1) Carotenes in oil (Smaco) were dissolved in Skellysolve, 2 cc of the latter containing approximately 10  $\gamma$  Curve IV in Fig 1 shows the color development of 10  $\gamma$  of these carotenes when 10 cc of standard reagent were added, in the experiment recorded in Curve V of Fig 1,

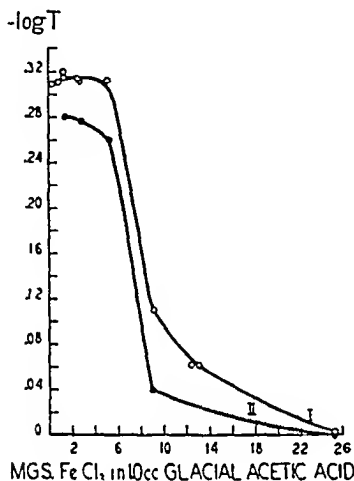


FIG 5

FIG 5 Curve I, 2 cc of Skellysolve containing 100  $\gamma$  of  $\alpha$ -tocopherol plus 5 cc of glacial acetic acid containing 5 mg of  $\alpha, \alpha'$ -bipyridine plus 5 cc of glacial acetic acid containing amounts of iron as indicated on the abscissa Maximum readings within 6 minutes were plotted Curve II, the same as for Curve I except that the Skellysolve contained both 100  $\gamma$  of  $\alpha$ -tocopherol and 90 mg of olive oil

FIG 6 Curve I, 2 cc of Skellysolve containing 100  $\gamma$  of  $\alpha$ -tocopherol plus 5 cc of glacial acetic acid containing 2.5 mg of  $\text{FeCl}_3$  plus 5 cc of glacial acetic acid containing amounts of bipyridine as indicated on the abscissa in mg of  $\alpha, \alpha'$  bipyridine in 10 cc of glacial acetic acid Maximum readings within 6 minutes were plotted Curve II, the same as for Curve I except that the Skellysolve contained both 100  $\gamma$  of  $\alpha$ -tocopherol and 90 mg of olive oil Curve III, percentage differences in extinctions between Curves I and II

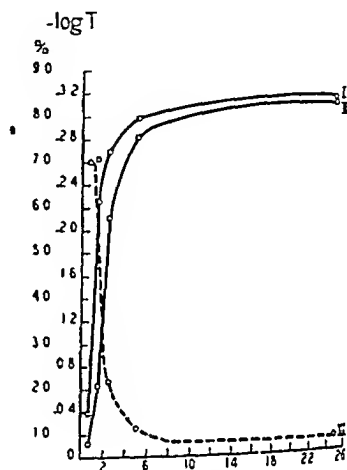


FIG 6

90 mg of olive oil were present, leading to depression and bleaching of the color

**Effect of Varying Ferric Ion and Bipyridine Ratios**—The explanation of results obtained with the iron-bipyridine reaction is complicated by an intricate system of equilibria involving ferric and ferrous ions, bipyridine, and fat Fig 5 demonstrates the fact that increasing ferric concentrations with constant ferrous ion and bipyridine content in the absence of fats lead to a rather sudden loss of color at a concentration of 5 mg of  $\text{FeCl}_3$  per

10 cc of glacial acetic acid, which corresponds to a ferrous-ferric ratio of approximately 1 100 and to a molar bipyridine- $\text{FeCl}_3$  ratio of 1 1. The ferrous bipyridine color disappears entirely at a bipyridine- $\text{FeCl}_3$  ratio of 1 5. However, there was very little effect upon the color when a 5-fold amount of  $\text{FeCl}_3$  was added after the development of the color

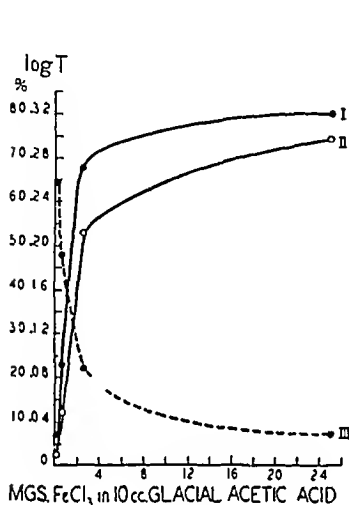


FIG 7

FIG 7 Curve I, 2 cc of Skellysolve containing 100  $\gamma$  of  $\alpha$ -tocopherol plus 10 cc of glacial acetic acid containing amounts of  $\text{FeCl}_3$  as indicated on the abscissa and twice the amount of  $\alpha, \alpha'$ -bipyridine. Maximum readings within 6 minutes were plotted. Curve II, the same as for Curve I except that the Skellysolve contained both 100  $\gamma$  of  $\alpha$ -tocopherol and 90 mg of olive oil. Curve III, percentage differences in extinctions between Curves I and II.

FIG 8 Curve I, 2 cc of Skellysolve containing 100  $\gamma$  of  $\alpha$ -tocopherol plus 10 cc of glacial acetic acid containing 2.5 mg of  $\text{FeCl}_3$  and 5 mg of  $\alpha, \alpha'$ -bipyridine. Curve II, 2 cc of Skellysolve containing 100  $\gamma$  of  $\alpha$ -tocopherol and 180 mg of purified rat fat plus 5 cc of glacial acetic acid containing 2.5 mg of  $\text{FeCl}_3$ , followed after 30 seconds by 5 cc of glacial acetic acid containing 5 mg of  $\alpha, \alpha'$ -bipyridine. Curve III, 2 cc of Skellysolve containing 100  $\gamma$  of  $\alpha$ -tocopherol and 180 mg of purified rat fat plus 10 cc of glacial acetic acid containing 2.5 mg of  $\text{FeCl}_3$  and 5 mg of  $\alpha, \alpha'$ -bipyridine.

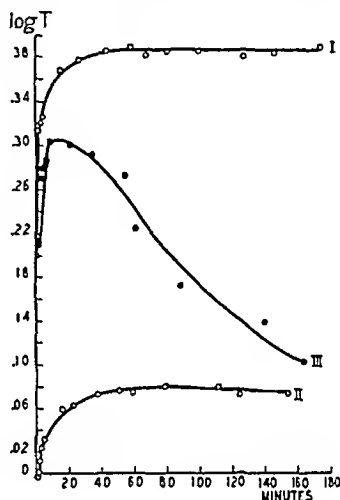


FIG 8

with the standard reagent, probably because of the stability of the ferrous bipyridine complex.

When either the bipyridine is increased with constant  $\text{FeCl}_3$  (Fig 6) or when both the  $\text{FeCl}_3$  and the bipyridine are increased at a ratio of 1 2 (Fig 7), the color quickly climbs to a maximum. It is to be noted that the standard reagent, as recommended by the laboratories of Merck and

Company (3), produces the maximum color with a minimum of bipyridine. Curves II in Figs 5, 6, and 7 indicate the influence of fats upon the respective equilibria. In each individual case, 90 mg of olive oil were present. One sees that, regardless of either the absolute concentration or the ratio of bipyridine and iron, the fat always exerts a depressing effect upon color development with the smallest percentage effect at high absolute bipyridine contents or at high bipyridine-ferric ion ratios. However, under the latter condition, in which the fat interferes least with the color intensity, test solutions frequently became clouded.

*Effect of Successive or Simultaneous Addition of Iron and Bipyridine*—The complexity of the equilibria involved is further illustrated by Curves II and III in Figs 2, 3, and 8. The only difference between the experiments demonstrated in these curves is the order in which the reagents were added. When iron and bipyridine are added simultaneously in the presence of fat, the maximum color is developed in 2 to 30 minutes, and thereafter it gradually bleaches. The rate of bleaching depends upon the character and concentration of the fat used, increasing with higher concentrations. The different effects of various fats are indicated in Curves III and IV of Fig 3, and Curve II in Fig 8, in which sesame oil, olive oil, and rat fat were used.

In Curves II of Figs 2, 3, and 8 are presented the results of experiments in which the bipyridine was added 30 seconds after all the other reactants had been mixed. It is to be noted that the final concentration of all substances was the same as that recorded in Curves III. The most plausible explanation of the fact that initially no color at all is developed is again the interaction of ferrous ions with the fat. From the rate of color development, the conclusion can be drawn that in the experiment recorded in Curve II of Fig 3 the stability of the fat-iron complex was less than that of the ferrous bipyridine compound, with a different fat, however (Fig 8), the opposite seemed true. The varying stability of the fat-iron complexes formed is demonstrated in Curve II of Fig 8, where the extinction never exceeded 21 per cent of that of a pure tocopherol solution, and in Curve II of Fig 3, where the extinction eventually reaches the same maximum as a fat-free solution.

When the ferric chloride solutions were added after the bipyridine to the fat-free solutions, the curves were the same as upon simultaneous addition of the reagent.

In Curves I and II of Fig 9, evidence is presented that not only the ferrous but also the ferric ion reacts in some manner with the fat. This is indicated by the rise from *a* to *b* on Curve I as compared to the rise of *a* to *b* on Curve II. In both cases, 67  $\gamma$  of  $\alpha$ -tocopherol were added. Quantitatively, the rise from *a* to *b*, Curve I, is of approximately the correct order for the amount of tocopherol added, while from *a* to *b*, Curve II, the

rise is only 28 per cent of that in the fat-free solution (Curve I) If the ferric ion, of which there is an excess in the reagent, were entirely free to react with bipyridine, a rise of the same order as the first one should be expected Further addition of bipyridine had no effect (compare Fig 6)

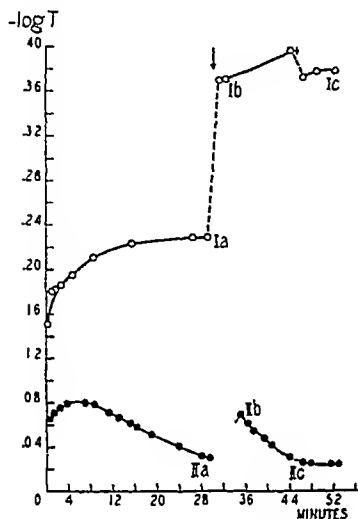


FIG 9

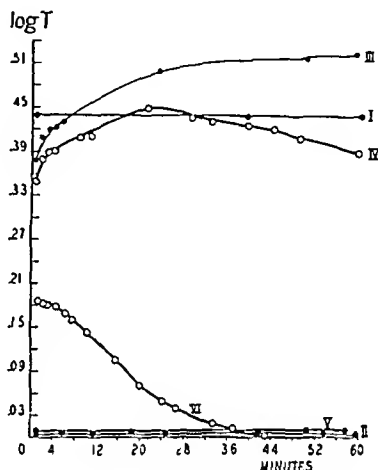


FIG 10

FIG 9 Curve I, a, 2 cc of Skellysolve containing 67  $\gamma$  of  $\alpha$ -tocopherol plus 10 cc of glacial acetic acid containing 2.5 mg of  $\text{FeCl}_3$  and 5 mg of  $\alpha, \alpha'$ -bipyridine, b, 1 cc of Skellysolve containing 67  $\gamma$  of  $\alpha$  tocopherol was added to a 30 minutes after the beginning of the experiment, c, 1 cc of glacial acetic acid containing 5 mg of  $\alpha, \alpha'$ -bipyridine was added to b 45 minutes after the beginning of the experiment Curve II, a, b, c, same as a, b, c of Curve I except that the Skellysolve contained 133 mg of an old sample of sesame oil

FIG 10 Curve I, 2 cc of Skellysolve plus 5 cc of glacial acetic acid containing 23  $\gamma$  of ferrous ion, followed after 30 seconds by 5 cc of glacial acetic acid containing 5 mg of  $\alpha, \alpha'$ -bipyridine Curve II, the same as for Curve I except that the Skellysolve contained  $80 \times 10^{-3}$  cc of redistilled hexadecane Curve III, 2 cc of Skellysolve containing 130  $\gamma$  of  $\alpha$ -tocopherol plus 10 cc of glacial acetic acid containing 2.5 mg of  $\text{FeCl}_3$  and 5 mg of  $\alpha, \alpha'$ -bipyridine Curve IV, the same as for Curve III except that the Skellysolve contained  $40 \times 10^{-3}$  cc of redistilled hexadecane Curve V, the same as for Curve I except that the Skellysolve contained 200 mg of albolene Curve VI, the same as for Curve III except that the Skellysolve contained 200 mg of albolene

*Color Depression and Physical Properties of Fats*—While of the fourteen different fats and oils examined all but tripalmitin caused both the color-depressing and the bleaching effect, no definite correlation between this



effect and the iodine numbers, melting point, or saponification numbers of the fats could be found. The absence of any correlation between iodine number and color-depressing effect is also substantiated by the fact that a completely hydrogenated sample of sesame oil gave a very strong effect (Fig 4), as did hexadecane, purified samples of albolene<sup>4</sup> (Fig 10), paraffin (m p 56-58°), and the unsaturated methyl arachidonate, whereas the completely saturated tripalmitin and ethyl laurate had no effect.

In Curve I of Fig 1, the results of an experiment are presented in which a sample of rat fat depressed the color by approximately two-thirds. A second sample of rat fat, purified exactly as the first except that in the course of the various operations air was replaced by nitrogen, depressed the color only by 5 per cent. With horse fat, sesame oil, and olive oil, similar differences between old and fresh samples were observed. However, when air was bubbled through olive oil for 3 hours at 50-60°, no increase of the effect could be observed.

### CONCLUSIONS

From the experimental results reported in this paper, the conclusion can be drawn that the color-depressing effect of fats on the reaction of tocopherol with iron-bipyridine is due to the formation of some kind of an iron-fat complex. The formation of the complex leads, according to the particular equilibria, to decreased ferrous bipyridine formation and thus decreases the color of a fat-containing solution as compared with a fat-free one.

Why the different fats and hydrocarbons studied have quantitatively different effects cannot yet be explained because of the intricate system of equilibria present and the lack of information about the type of the complexes formed.

### SUMMARY

1 The presence of glycerol, oleic acid, acetone, alcohol, or benzene, changes of temperature, and exposure to light do not affect the depressing effect of fats on the iron- $\alpha, \alpha'$ -bipyridine reaction of tocopherols.

2 Fats and oils also depress the iron-bipyridine color if carotenes are used as reducing agents.

3 The depressing effect is observed also in the absence of tocopherol or carotenes, when ferrous ion is exposed to the action of fat, especially if the ferrous ion is added to the fat before the bipyridine.

4 Peroxide-free and hydrogenated fats, as well as saturated hydrocarbons, also give the effect. No color depression is noted with tripalmitin and ethyl laurate.

<sup>4</sup> Albolene is a mixture of hydrocarbons from approximately  $C_{15}$ - $C_{25}$  with zero iodine number.

5 The color-depressing effect of fats and hydrocarbons on the reaction of iron with bipyridine points to some kind of interaction between fats or hydrocarbons and ferrous and ferric ions

6 The ferrous bipyridine reaction is complicated also in the absence of fats by an intricate system of equilibria involving the concentration and the ratios of bipyridine and ferrous and ferric ions. Different fats and hydrocarbons studied have qualitatively different effects for which a correlation with the physical properties has not as yet been found

We are greatly indebted to Dr. Hans T. Clarke for his interest, suggestions, and criticism in this work.

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# THE DEPOSITION OF ANTIOXIDANTS IN THE ABDOMINAL FAT DEPOTS\*

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Data reported in an earlier paper (1) strongly suggest that the antioxidants responsible for the normal stability of the body fat of the rat and derived solely from dietary sources. Further, the stability of fat produced by rats receiving a vitamin E-deficient diet is very low. These facts have been utilized by us in an attempt to determine whether or not certain well known *in vitro* antioxidants are deposited in adipose tissues. In the earlier studies (1) yeast and hydroquinone were found not to increase the stability of body fat when added to a vitamin E-deficient diet, only  $\alpha$ -tocopherol was shown to restore a "normal" stability to the fat. The object of this investigation has been to study the deposition of other antioxidants in the body fat of low stability in vitamin E-deficient rats.

## EXPERIMENTAL

From the time of weaning, rats were given a vitamin E-deficient diet of the following percentage composition: casein 19.1, sucrose 52.5, yeast 5.0, salts 4.3 (modified (2)), and lard 19.1 (rancidified to a peroxide value of 20 or more). 1 drop of vitamin A concentrate containing approximately 500 IU was given every 2 weeks. The mothers of these rats had been given a deficient diet and received just enough vitamin E to bear their litters. The experimental rats were 100 to 150 days old and weighed about 175 gm. Many of the rats showed some dystrophic paralysis of the hind limbs.

The following substances were selected as readily available and relatively pure compounds with antioxidant properties:  $\alpha$ -tocopherol (3), ascorbic acid (4),  $\gamma$ -tocopherol<sup>1</sup> (3), lecithin (5),  $\alpha$ -naphthol (6), hydroquinone (3, 4), and nordihydroguaiaretic acid (NDGA) (7).

In groups of five, matched for weight, the rats were fed the test com-

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† The experimental data are taken from a thesis submitted to the Faculty of the Graduate School of the University of Minnesota by H T Hanson in partial fulfillment of the requirements for the degree of Master of Arts.

<sup>1</sup> The  $\gamma$ -tocopherol employed in these studies was generously supplied by Merck and Company, Inc.

pounds in amounts which were molar equivalents of a 50 mg dose of  $\alpha$ -tocopherol. The compounds were dissolved in the ethyl esters of lard fatty acids completely free of unsaponifiable matter, so that the dose for each rat was contained in 1.0 ml. The calculated amounts of ascorbic acid, hydroquinone, and NDGA did not dissolve in the esters, but the excess was fed as a suspension. Control animals were given 1.0 ml of the lard esters alone. Administration was by stomach tube, the rats were sacrificed 6 days later, and the perirenal and perigonadal fat was promptly rendered as described in an earlier publication (1). Lundberg *et al* (8) have shown that the maximum deposition in abdominal fat in response to a single dose of  $\alpha$ -tocopherol occurs between the 7th and 10th days after administration. What happens to the tocopherol during the interval is

TABLE I  
*Stabilities of Rendered Abdominal Fats from Rats Fed Various Antioxidants*

Group No (1)	Compound (2)	Amount fed (3)	Induction period	
			By O absorption at 100° (4)	By peroxide accumulation at 63° (5)
		mg	min	days
1	Control		31	1.5
2	$\alpha$ -Tocopherol	50	418	21.0
3	Ascorbic acid	20	17	1.2
4	$\gamma$ -Tocopherol	50	211	13.0
5	Lecithin (soy bean)	85	25	2.0
6	$\alpha$ -Naphthol	17	23	1.0
7	Hydroquinone	13	42	1.5
8	Nordihydroguaiaretic acid	28	22	1.1

not known, but great increases in blood levels in man have been reported (9) to follow oral administration. In addition to increased blood content, temporary storage in the liver and other viscera should be considered.

Stability of the rendered fat was determined by measuring the rate of oxygen absorption of 0.2 ml samples in a Warburg respirometer at 100°, as described by Lundberg *et al* (8). Values given in Column 4 of Table I represent the length of the induction period in minutes. Each value represents at least three determinations for the fat obtained from five rats. Groups 1, 2, and 4 have been repeated with additional groups of rats. In all cases these values were comparable to estimates of stability obtained by measuring the accumulation of peroxides in 1.0 ml samples stored in small glass vials in an oven at  $63^\circ \pm 0.5^\circ$ . The end of the induction period was estimated from the break in the peroxide-time curve. This usually occurred at a peroxide value of about 20.

## DISCUSSION

Examination of the data at once reveals that only the tocopherols had any significant effect upon the stability of the fat. There are three principal considerations if one attempts to explain the failure of the other five antioxidants: (a) Failure of absorption is probably not a factor, except possibly in the case of NDGA, which is not known to be completely absorbed. (b) After absorption, these compounds may be immediately metabolized or detoxified. This is a highly probable fate for three of the antioxidants fed.  $\alpha$ -Naphthol is reported (10) to be excreted in the urine as a glucuronate. Hydroquinone appears in the urine as an ethereal sulfate (11). Lecithin is readily hydrolyzed in both the intestinal tract and the body tissues. Hence it is not likely that these compounds survive unchanged for long in the blood stream. (c) After absorption, these compounds may not be stored in the fatty tissues. Here the best predictions must be based on solubilities. The amount of water in adipose tissues being very small, it is doubtful that much storage of fat-insoluble compounds would occur. Ascorbic acid and hydroquinone are negligibly soluble in lipids. Preliminary chemical tests made in this laboratory failed to reveal ascorbic acid in the adipose tissue either free or in the esterified form. Although  $\alpha$ -naphthol and lecithin are fat-soluble, their elimination is strongly indicated. NDGA also is somewhat fat-soluble and hence its disposition is more devious. According to Lundberg, Halvorson, and Burr (7), NDGA is an effective antioxidant in concentrations of 0.01 per cent in lard. To obtain that concentration in the abdominal fat (approximately 4 gm.) only 0.4 mg. (one-seventieth of the dose) would have to be deposited there. Hence this test is sensitive enough to justify the conclusion that no NDGA reached the fat depots. If the compound was absorbed, it may have been immediately excreted. An additional factor to be considered is that ascorbic acid and lecithin have little antioxidogenic action other than as synergists to the phenolic inhibitors.

The specificity demonstrated in this stability test is similar to that described by Evans *et al.* (12) who, in reporting forty effective compounds among 130 tested for vitamin E activity, suggest three requirements necessary for a compound to show such activity. These requirements may be the same as those which determine whether or not an ingested antioxidant will be stored in the adipose tissue: (a) a structural skeleton which can readily be converted in the body to an oxidation-reduction system with a potential in a specific range, (b) accessory groups conferring solubility properties specific for absorption and transference to the place where it is to be used, (c) that the chemical transformations necessary for (a) be among the known biological transformations (cleavage, hydrolysis, dehydration, reduction, etc.).

Although Mason (13) and Hines and Mattill (14) have suggested the

liver as the principal storage organ for vitamin E in rats, recent work by Lundberg *et al* (8) has shown that the abdominal fat may equal or surpass the liver in storing tocopherols. They report that the ingestion of only 1 mg of  $\alpha$ -tocopherol significantly increased the keeping time of fat from deficient animals. Also, chemical analysis of abdominal fat from rats after ingestion of 500 mg of  $\alpha$ -tocopherol (8) indicated a minimum deposition of 97  $\gamma$  per gm. This value is more than twice the value (42.3  $\gamma$  per gm) obtained for the liver of animals receiving 100 mg daily, as determined by Hines and Mattill (14). It should be kept in mind that commercially rendered lard contains little tocopherol. In these experiments large amounts of tocopherols were fed and the rendering process was designed to conserve tocopherols. Hence no support for species differences can be derived from comparisons of these values with the tocopherol content of commercial lards.

The data presented above indicate also a great difference between the tocopherol isomers, the  $\alpha$  compound having almost twice the effect of the  $\gamma$  isomer. This difference is the more impressive when one considers that  $\gamma$ -tocopherol is about 3 times as active an antioxidant as  $\alpha$ -tocopherol when added to lard *in vitro* (3). Only one-sixth as much of the  $\gamma$  isomer as  $\alpha$  isomer in the fat would be required to produce this effect.

These two isomers, therefore, differ in this test in the same direction as they do in antisterility functions, in the latter,  $\alpha$ -tocopherol is 12 times as effective as  $\gamma$ -tocopherol (15).

There is some circumstantial evidence to be gained from the preceding discussion in favor of a theory that tocopherols act in the body as inhibitors of oxidative reactions. Other support might be derived from reports that the injection of rancid fat produces symptoms resembling those of vitamin E deficiency (16), that the oxygen consumption of vitamin E-deficient tissue is increased above the normal but is reduced by  $\alpha$ -tocopherol phosphate (17), and that tocopherol inhibits the destruction of carotene by water extracts by minced rat stomachs (18).

#### SUMMARY

This work confirms our earlier report (1) in showing that the keeping time of the body fat from rats fed a vitamin E-deficient diet is very low, practically lacking an induction period.

Addition to the diet of five antioxidants other than tocopherols (ascorbic acid, hydroquinone,  $\alpha$ -naphthol, nordihydroguaiaretic acid, lecithin) does not increase the stability of the fat.

The  $\alpha$  and  $\gamma$  isomers of tocopherol greatly prolong the keeping time of the rendered fat when fed to rats which were previously vitamin E-deficient.

This evidence strongly suggests that only compounds having the properties of vitamin E are deposited in adipose tissue as effective antioxidants

Comparison of the effectiveness of the isomers as *in vitro* antioxidants and as antioxidants stored in adipose tissue after ingestion seems neither to confirm nor deny the possibility of a metabolic function of the tocopherols as inhibitors of oxidative reactions. The differences shown may be a clue to the elucidation of such functions.

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# A METHOD FOR THE QUANTITATIVE DETERMINATION OF HEMOGLOBIN IN TISSUES\*

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A reliable and simple analytical method for the estimation of hemoglobin in tissues has not been available, and studies of lung edema, to be reported elsewhere, required such a method. The procedures of Flink and Watson (1) for assay of hemoglobin in feces have been adapted to the analysis of hemoglobin in several tissues, and the method has been found to be convenient and reliable.

Flink and Watson, in a procedure described for the quantitative hemoglobin determination in feces, converted hemoglobin first to acid hematin, then to alkaline hematin, and finally to pyridine ferrohemochromogen. The latter was measured quantitatively.

We have adapted their methods to the determination of hemoglobin in tissues and have modified their procedures as follows: (1) by extracting the tissue repeatedly and allowing the final extraction to go on for at least 12 hours, a more complete, and thus more uniform analysis has been achieved, (2) by carrying out the final extraction in the cold, (3) by converting the tissue hemoglobin to acid hematin and determining its concentration colorimetrically, instead of going on to convert this in turn to pyridine ferrohemochromogen, (4) by developing technical refinements to make the technique suitable for accurate results in the determination of tissue hemoglobin.

By adding known amounts of hemoglobin to the tissues assayed, the reliability of this method for tissue hemoglobin analysis has been tested to give satisfactory recoveries.

## Reagents—

Acetone, C P

Alcohol-ether mixture consisting of ether, U S P X, and 95 per cent ethyl alcohol in the proportion of 4 parts to 1.

Acid solution consisting of equal quantities of 5 per cent HCl and glacial acetic acid.

## Procedure

Tissue samples weighing between 2 and 4 gm. are thoroughly minced with a pair of scissors and then extracted with about 8 cc. of acetone. After

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centrifugation for about 2 minutes at 3500 R P M, the acetone is decanted and 3 cc of the acid solution are added to the minced tissue. The latter is then extracted repeatedly with alcohol-ether until the tissue is white. The final extraction is carried out overnight in the icebox at 5°. The alcohol-ether extract is also kept at 5° for 12 hours and then filtered into a 100 cc volumetric flask through a quantitative filter paper. The alcohol-ether mixture is poured over the filter paper to remove all the pigment and the volume is brought up to 100 cc. A clear solution of ethereal acid hematin results, the absorption of which is determined at wave-length 635  $m\mu$  in the Evelyn colorimeter.

Special care must be taken to avoid evaporation of water in weighing the samples. Acetone is poured over the weighed sample as soon as possible because drying of the tissue makes subsequent extraction difficult and may result in large errors in the final values. The mincing of the tissues is then completed in the acetone solution, with the use of a rubber policeman to remove the tissue from the scissors.

### Results

Hemoglobin recovery from dog blood was studied and a straight line relationship was obtained between the log of the galvanometer deflection and the amount of hemoglobin in the solution. Dog lung tissue, to which increasing amounts of dog blood were added, also gave a similar relationship, with hemoglobin recovery from pure blood as the basis for determining the amount of hemoglobin in the lung tissue to which no blood had been added. The  $K$  value of the slope of this straight line was 213, i.e., the product of the log of the galvanometer deflection times 213 gave the value in mg for the hemoglobin present in the weighed tissue sample analyzed. In the case of dog heart tissue,  $K = 214$ . Fig 1 shows the values 2 - log of the galvanometer deflection for the corresponding  $L$  values in the case of beef blood added to bovine lung tissue. The fact that the straight line intersects at the origin indicates the absence of turbidity in the solution.

The calculation used in determining these values was as follows

$$X = \frac{(100L) \times K \text{ (for particular tissue)}}{W}$$

$L = 2 - \log$  of galvanometer deflection

$K =$  slope of straight line of hemoglobin recovery plotted against galvanometer deflection for any one tissue

$W =$  weight (gm) of sample of tissue analyzed

$X =$  mg of hemoglobin per 100 gm of tissue

Table I shows the results of aliquot determinations of hemoglobin on lung, heart, and liver tissues of different species. The maximum difference of hemoglobin values between repeated sampling is 6.4 per cent for the analyses shown.

It was found that for lung tissue and heart tissue determinations, the optimum weight of the samples was between 2 and 4 gm, and for liver between 2 and 3 gm. It might be said that the narrower the range between weighed samples, the greater will be the uniformity of the results obtained,

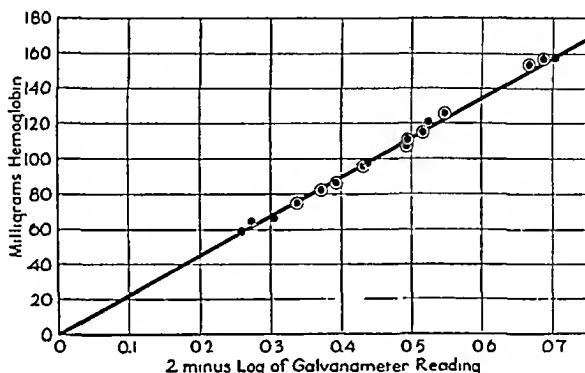


FIG 1 Hemoglobin recovery from lung tissue. The dots represent recovery from lung tissue alone. The circled dots represent recovery from hemoglobin added to lung tissue. Data collected from three separate experiments.  $K = 213$

TABLE I

*Aliquot Samples of Lung, Heart, and Liver Tissue*

The results are expressed in mg of hemoglobin per 100 gm of tissue

Tissue	Experiment No	Aliquot 1	Aliquot 2	Aliquot 3	Aliquot 4
Beef lung	1	1672	1637	1639	1626
	2	1615	1614		
	3	1816	1945		
Goat "	1	2354	2199	943 5	1104
	2	1876	1862		
Dog Heart	1	897 4	937 6	1106	1104
Goat "	1	1155	1171		
Beef liver	1	1641	1636		
	2				

although within the range of the weights given we were able to obtain a maximum difference of 8 per cent between any two samples

It is also necessary to note that the range of values reported does not cover the maximum ranges for a given tissue even in the same species. Goat lung, for instance, with which we have worked most extensively, showed values for hemoglobin varying between 900 and 4000 mg per cent

## DISCUSSION

In chloride space studies on animal tissues, determination of the blood content of the tissues is a difficult procedure. In many studies this value has been either assumed or neglected. Lowry and Hastings (2) recently reported difficulty with turbidity in studying muscle hemoglobin content and sought to avoid this difficulty by use of the spectrophotometer. Our attempts to adapt Whipple's (3) method of determination of muscle hemoglobin to the quantitative study of lung hemoglobin proved unsuccessful. The above method has proved to be useful in estimating the residual blood in lung tissue.

## SUMMARY

A colorimetric method for the quantitative determination of hemoglobin in lung, heart, and liver tissue is described and tests of its reliability reported.

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# AN INOSITOLLESS MUTANT STRAIN OF NEUROSPORA AND ITS USE IN BIOASSAYS

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Among the mutant strains of *Neurospora crassa* isolated according to the technique of Beadle and Tatum (1) following treatment with x-rays or ultraviolet light are five which require a supplement of inositol for normal growth. If the possibility of contamination is disregarded, these mutant strains are of independent origins. All were derived from ascospores following matings in which one parent was treated with ultraviolet light in the conidial stage. They are designated as Strains 37102, 37401, 46316, 46802, and 64001.

These five strains are presumed to represent recurrences of mutation in a single gene, because they show similar quantitative responses to inositol and because in tests for allelism by the heterocaryon method (2) growth responses were negative. In addition, crosses between the two strains, Nos 37102 and 37401, gave only mutant progeny.

Crosses between Strain 37401 and the original wild type strain, which does not require inositol for normal growth, have repeatedly yielded asci in which four ascospores gave cultures requiring inositol for growth and four gave cultures like the original wild type. In view of the mechanism of ascospore formation in *Neurospora* (3), such observations constitute valid evidence that the mutant strain differs from the original in a single localized part of one chromosome, presumably in one gene modified through inactivation or loss. Heterocaryons with other mutants such as *pantothenicless* show that at least under certain conditions this *inositolless* mutant gene is recessive to its normal allele ((2), H. Grant, unpublished).

*Inositolless* mutant strains show essentially no growth in media free of inositol. With inositol supplements their growth is a function of inositol concentration over a range of about 5 to 40  $\gamma$  of inositol per 20 ml of medium. It is the primary purpose of this paper to report studies indicating that an *inositolless* strain of *Neurospora* can be used as a basis for estimating inositol quantitatively in a manner essentially similar to that followed by Woolley (4) and by Williams *et al* (5) using yeast strains as a test organism. Unless otherwise stated all results reported are based on the use of Strain 37401.

## Methods

The basal medium used was the same as that previously reported (6) and has the following composition, in gm per liter ammonium ta.

ammonium nitrate 1, potassium dihydrogen phosphate 1, magnesium sulfate ( $7\text{H}_2\text{O}$ ) 0.5, sodium chloride 0.1, calcium chloride 0.1, sucrose 20.0, biotin  $5 \times 10^{-6}$ . Trace elements are added as salts as follows, in mg per liter: B 0.01, Cu 0.1, Fe 0.2, Mn 0.02, Mo 0.02, Zn 2.0. In some experiments the basal medium given above was supplemented with 2.5 gm of asparagine per liter. While this increases dry weight attained at high inositol levels, it has little or no effect at those levels at which inositol is clearly limiting and at which bioassays are carried out.

Growth responses were determined by weighing dried mycelia (6). A study of the influence of flask size on variability of dry weights was made with 50, 125, and 250 ml Erlenmeyer flasks containing 10, 20, and 25 ml of medium respectively. The amount of inositol supplied was held constant at 20  $\gamma$  per flask. Under these conditions the average yields based on nineteen replicates of each size and determined after 68 hours were 17.6, 19.3, and 24.0 mg of dry mycelium per culture of the three sizes indicated. Variability was observed to increase somewhat with flask size. Although either 50 or 250 ml flasks will serve the purpose, 125 ml flasks were chosen as being generally most satisfactory.

Inoculations were made by adding 1 drop of conidial suspension to each flask. Because it was observed that the variability in yield between series inoculated at different times is greater than that within a single series, several tests of the effect of inoculum size on yield were made. These showed that, in general, when the inoculum is varied from about  $100$  to about  $6.5 \times 10^6$  conidia per culture (10 ml of medium supplemented with 20  $\gamma$  of inositol in a 50 ml flask), dry weights after 2 days are greater with a large inoculum, but after 4 or 5 days are greater with a smaller inoculum. Since it is not practicable to control accurately effective inoculum size from day to day, it is evident that a control series with known amounts of inositol must be run with each series of cultures. It is recommended that inocula be roughly standardized at a low level and that conidia be taken from stock cultures of a uniform age of 4, 5, or 6 days.

All growth experiments were carried out at  $25^\circ$ . At this temperature satisfactory results are obtained for Strain 37401 in assays for inositol by allowing growth to take place for approximately 72 hours. Less variability is found if cultures are not shaken (Dr. David Bonner, unpublished). Mycelia are removed from flasks, the free liquid pressed out, and the pads weighed following drying to constant weight at  $80-90^\circ$ .

Natural products on which sample assays were made were hydrolyzed by autoclaving in 3 per cent sulfuric acid for 2 hours at 15 pounds (6) or by refluxing with 18 per cent hydrochloric acid for 6 hours (4). In the one comparison available more inositol appears to be released during the second procedure than with the first, as shown by the fact that dry brewers' yeast

gave a value of 2.4  $\gamma$  of inositol per mg after hydrolysis with sulfuric acid and 4.6  $\gamma$  per mg after hydrochloric acid hydrolysis. Following both methods, reproducibility and recoveries at two levels of material were satisfactory. This indicates that free inositol is measured by the method with reasonable accuracy but that the two methods of hydrolysis differ in the extent to which the active compound is freed. Actually it is known that the complete hydrolysis of phytin, the principal reservoir of inositol in plants, is difficult to bring about (Table I) (7, 8) and it is therefore suggested that a special study of methods is necessary if one wants to be certain that all bound inositol in a particular material being assayed is liberated.

### Results

*Specificity of Mutant Strains*—It has been found that a *pyridoxineless* strain of *Neurospora sitophila* and an *arginineless* strain of *Neurospora crassa* are able to grow on a basal medium if the pH is raised and other conditions are favorable (9, 10). Tests of Strain 37401 on regular basal medium lowered from pH 5.5 to 4.6 with HCl or raised to 6.8 with NaOH showed that these modified media do not support growth. Replacing the ammonium ion with potassium, thus leaving nitrate as the sole nitrogen source, likewise did not alter the growth response in the absence of inositol. This nitrate medium was tested at pH 4.5 and 7.0. Schopfer's medium in which asparagine serves as the nitrogen source does not support growth of the *inositolless* strain at pH 5.8. Lowering the temperature to 20° or raising it to 30° does not change the response on the basal medium.

The growth responses of all five strains of *inositolless* to inositol, phytin, and lipositol (11) were tested qualitatively and found to be similar. All grew normally on inositol but none grew on phytin or lipositol at a level of 400  $\gamma$  per 20 ml. It would therefore appear that *inositolless* strains are unable to hydrolyze the two latter compounds at a rate sufficient to initiate growth.

*Standard Curve*—The dry weights of mycelia obtained at different levels of inositol in two different experiments are shown in Fig. 1. These two curves illustrate extremes of variability. Assay values and recoveries in the experiments in which they were used were satisfactory for both curves, although the slopes are quite different in those regions of the curves useful in assay purposes and the range of concentrations over which they can be used differs rather widely. Presumably these differences are due to inoculum size, since it was shown that within a single series in which only inoculum size was varied the 3 day yield at 20  $\gamma$  of inositol per flask varied from 14.0 to 37.2 mg per culture. In any case, it is obvious that a standard curve must be determined for each series.

An observation of some interest is that at levels below 10  $\gamma$  per flask



the mycelia tend to stick to the bottoms and the sides of the culture flasks. This behavior has not been observed with other mutants under conditions of similar limited growth. Partial inositol deficiency evidently has some specific effect on the morphological pattern of the organism.

*Sample Assay Values*—Determinations of inositol content of a number of natural materials are summarized in Table I. It is seen that, in general, values obtained at different levels of the material being assayed are in reasonable agreement. Recovery experiments likewise give satisfactory results in most instances. With yeast extract, corn-meal, and solids from corn steep liquor there is a tendency for values to fall off as higher levels of material are used.

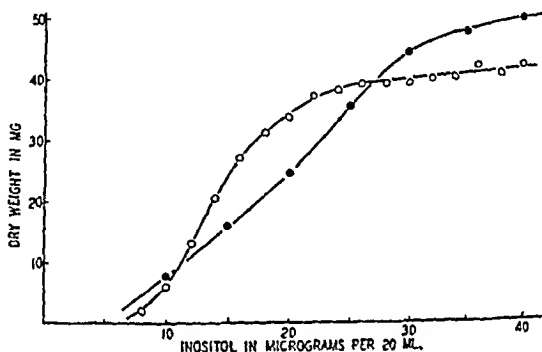


FIG. 1. Relation between the growth of an *inositolless* strain of *Neurospora* and the amount of inositol supplied in the medium. Based on two experiments carried out at separate times.

That unhydrolyzed corn steep liquor contains inositol in unavailable form is shown by the fact that the assay value increases from 4.4 to 10.2  $\gamma$  per mg after hydrochloric acid hydrolysis (Table I). Even this treatment may not free all inositol, since phytin gave only about one-third the calculated yield of inositol following hydrochloric acid hydrolysis (Table I).

#### DISCUSSION

As compared with the yeast growth methods of Woolley (4) and of Williams *et al.* (5), the *Neurospora* method has an advantage in that a simpler basal medium is used, and a zero growth response is obtained on this medium. Whether it has other advantages can only be determined by direct comparisons on a variety of materials. Its sensitivity is not as great as might be desired, although by supplementing the basal medium with a suitable increment of inositol 5  $\gamma$  or less can be measured by the method, with reasonable accuracy. The range of concentrations which

TABLE I  
Assays and Recovery Experiments with Various Natural Products

Material	Method of hydrolysis (see text)	Amount used	Inositol added	Inositol found		Inositol calculated per flask
				Per flask	Per mg material	
		mg	γ	γ	γ	γ
Phytin	HCl	0 16	0	11 0	145 0	35 2
Difco yeast extract, dry	None	4 0	0	17 2	4 3	
		6 0	0	24 0	4 0	
		8 0	0	30 2	3 8	
		2 0	10	18 5		18 0
		3 0	10	22 5		22 0
Spray-dried cereal malt syrup	"	4 0	10	24 8		26 0
		30 0	0	13 5	0 45	
		40 0	0	18 5	0 46	
		10 0	10	14 0		14 5
		20 0	10	18 0		19 0
Potato (oven-dried)	H <sub>2</sub> SO <sub>4</sub>	8 9	0	11 0	1 24	
		13 3	0	15 2	1 15	
		22 2	0	24 5	1 10	
		4 4	10	16 1		15 1
		8 8	10	21 2		20 2
Egg yolk (oven-dried)	"	13 3	10	25 2		25 3
		20 0	0	12 0	0 60	
		25 0	0	13 5	0 54	
		10 0	0	14 0		15 7
		20 0	0	17 0		21 4
Brewers' yeast, 8% moisture	"	3 0	0	7 0	2 3	
		5 0	0	12 2	2 4	
		2 0	10	15 5		14 7
		4 0	10	19 0		19 6
	HCl	3 0	0	14 3	4 7	
		5 0	0	22 5	4 5	
		2 0	10	18 0		19 2
		4 0	10	29 5		28 4
Corn-meal, yellow, 8% moisture	"	12 0	10	13 5	0 29	
		20 0	10	14 5	0 22	
		40 0	10	16 5	0 16	
<i>Neurospora</i> mycelium, wild type, 4% moisture	"	4 0	0	9 0	2 2	
		1 0	10	12 2		12 2
		2 0	10	13 2		14 4
		4 0	10	15 0		18 8
Dried whole milk, 3% moisture	"	20 0	0	12 5	0 63	
		40 0	0	19 0	0 48	
		12 0	10	16 0		16 6
		20 0	10	20 5		21 0
Corn steep liquor solids, 9% moisture	"	2 0	0	20 5	10 2	
		1 0	10	21 0		20 2

TABLE I—Concluded

Material	Method of hydrolysis (see text)	Amount used	Inositol added	Inositol found		Inositol calculated per flask
				Per flask	Per mg material	
Corn steep liquor solids	None	mg	γ	γ	γ	γ
		2 0	0	10 5	5 2	
		3 0	0	13 2	4 4	
		4 0	0	16 0	4 0	
		5 0	0	19 5	3 9	
Same as above, adsorbed with permutit and Amberlite IR-4*	"	6 0	0	23 0	4 7	
		2 0	0	10 0	5 0	
		3 0	0	13 5	4 5	
		4 0	0	16 5	4 1	
		5 0	0	20 0	4 0	
		6 0	0	23 0	4 7	
		0 5	10	12 8		12 2
		1 0	10	14 8		14 5
		2 0	10	20 0		19 0

\* Solution containing 20 mg of solids per ml run through an adsorption column of permutit and Amberlite IR-4. These adsorbents were shown in separate experiments not to adsorb inositol.

can be worked with is likewise somewhat narrower than one might wish. It is necessary that the amount of inositol per flask fall between approximately 10 and 30 γ, depending to some extent on the standard curve obtained.

The reliability of the method can be measured in several ways. A rough comparison with the method of Woolley (4) can be had by noting the values obtained for the same materials. For brewers' yeast, powdered whole milk, and corn-meal these values are 5.0, 0.57, and 0.24 γ per mg of oven-dry material by the *Neurospora* method as compared with values given by Woolley of 5.0, 0.5, and 0.5. It should be noted, however, that different samples of the materials assayed were used. Values obtained at different times by the *Neurospora* method for the same material are in good agreement.

The error variance of determinations of dry weights of mycelia has been determined by an analysis of variance made in connection with an experiment involving 109 cultures. Nineteen different levels of inositol, yeast extract, or yeast extract plus inositol were included. The error variance was 0.16 mg, which is equal to a standard error of 0.4 mg. This indicates that about two-thirds of the individual cultures of a series set up under similar conditions would be expected to yield dry weights within 0.4 mg of the true mean. At a level of 20 γ of inositol, about the midpoint of the standard curve, this corresponds to approximately 0.3 γ of inositol. The indicated precision is high for a bioassay.

An *inositolless* strain will be supplied directly to investigators who desire to make use of it in carrying out assays for inositol or in other investigations. In addition, Strain 37401 has been supplied to the American Type Culture Collection, School of Medicine, Georgetown University, 3900 Reservoir Road, N W, Washington, D C, and it can be obtained there.

The work reported here was supported by grants from the Rockefeller Foundation, the Research Corporation, and the Nutrition Foundation, Inc. Mutant Strain 37401 was detected and determined as *inositolless* by Miss Janet M. Wallace. The cross between Strains 37102 and 37401 was made and analyzed by Miss Agnes J. Vincenti. The author is indebted to Dr. D. W. Woolley for a sample of lipositol and to members of the Genetics Laboratories at Stanford University for helpful suggestions.

#### SUMMARY

A mutant of *Neurospora crassa* requiring inositol for growth is described. Its use in a bioassay method for inositol has been investigated. The suggested method appears to be satisfactory in reproducibility and precision. It is useful for concentrations between 5 and 30  $\gamma$  of inositol per 20 ml of medium. Inositol contents of a number of materials are reported, but it has not been established that these values represent both free and combined forms of inositol.

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# OBSERVATIONS ON THE ESTIMATION OF DESOXYRIBOSE NUCLEIC ACID

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Recent studies on the biochemistry of the nucleic acids have been reviewed by Mirsky (1). The key rôle of the desoxyribose type of these substances in the reproduction of inheritable characteristics has been emphasized by the identification of desoxyribose nucleic acid (DNA) as a major component of chromosomal nucleoprotein. More recently it has been demonstrated that this type of nucleic acid, isolated from encapsulated Type III pneumococci, will promote the transformation of non-encapsulated Type II pneumococci to the encapsulated Type III organism (2). DNA has also been demonstrated in some viruses, such as the elementary bodies of vaccinia (3) and an *Escherichia coli* bacteriophage<sup>1</sup>.

Increasing interest in problems of pathological phenomena of reproduction, such as uncontrollable cellular division and virus multiplication, requires that a knowledge of the metabolism of nucleic acid and methods of estimation of the components of this complex substance be advanced as rapidly as possible. In the course of attempts to identify the nucleic acid of a hitherto incompletely characterized infectious agent, it soon became apparent that other components of the preparation markedly interfered with the few methods available for the identification of nucleic acid. This has made necessary the reexamination of some of these methods and the introduction of new reagents and techniques.

Desoxyribose, the sugar present in DNA, as contrasted to ribose in the nucleic acid of cytoplasmic particles, some plant viruses, etc., has accounted for the specificity of numerous colorimetric reactions used in distinguishing these materials. Three of these reactions have been adapted for quantitative estimation, namely the Feulgen (4), carbazole (5), and diphenylamine (6) reactions. The Feulgen reaction is given non-specifically by aldehydes, and its use is contraindicated unless special precautions have been taken to eliminate these materials. The carbazole reaction is markedly unspecific, since the use of strong sulfuric acid converts most

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<sup>1</sup> Cohen, S. S., and Anderson, T., unpublished data.

carbohydrates into products which will form some colored substance with this reagent. It is of interest that the concentrated acid used will enable at least a partial hydrolysis of pyrimidine desoxyribosides (6). In that connection it is to be emphasized that neither the Feulgen nor the diphenylamine reaction, as commonly employed, permits the estimation of pyrimidine desoxyribosides. The latter reaction is the most specific of those hitherto developed, and has been used exclusively by various workers for purposes of estimation.

Seibert (7) varied this method slightly to account for color due to impurities in the solution to be analyzed and Dounce (8), in using the diphenylamine reaction on nuclear material, took additional precautions in minimizing turbidities in the colored solution. The elimination of turbidities by means of preliminary enzymatic hydrolysis and subsequent isolation procedures, before estimation, has been employed for tissue (6) and vacuolar elementary bodies (3). The types of substances which give reactions other than the blue color characteristic of desoxyribose are not unknown in biological materials and have been considered by Pine (9). Furthermore, certain anomalous purple reactions, given by some carbohydrate-containing materials, may well obscure or otherwise be mistaken for a positive diphenylamine reaction\*. In view of these considerations, it should be apparent that the estimation or even identification of DNA in complex materials is not necessarily a simple matter, and statement as to the presence or absence of this compound is inadmissible without the presentation of data (e.g., amounts of samples used, volume of reagents, controls employed, etc.) which led to the conclusions presented. The desirability of the use of additional reactions in the identification and estimation of DNA should therefore be clear.

#### EXPERIMENTAL

The observation was made that, preliminary to the estimation of phosphorus, a red color was produced in the perchloric acid digestion of some materials suspected to contain DNA, and that this color was increased by the addition of tryptophane†. Subsequent investigation demonstrated

\* It has been noted, for instance, that the isolated cytoplasmic particles of the normal allantoic membranes of fertile hens' eggs give a purple color in the diphenylamine reaction. In as much as these particles possess a range of sedimentation constants which would result in their inclusion in preparations of influenza virus under conditions of cellular destruction, liberation into allantoic fluids, and isolation by means of differential centrifugation, it would be most difficult to interpret a weakly positive Dische reaction (10) as strong evidence for the presence of DNA in influenza virus.

† It has been pointed out that most desoxyribonucleoproteins contain very small amounts of tryptophane (1), a fact which has certainly limited the probability of this reaction being observed previously.

that this color, which was due to the presence of desoxyribose in these materials, was produced more specifically and controllably in perchloric acid than in the concentrated hydrochloric acid used by Thomas (11) in the condensation of sugars and aldehydes with tryptophane, although the colors eventually produced as a result of condensation were the same with either acid. Color development, because of condensation of the desoxyribose from 0.1 mg of DNA per cc with tryptophane in 30 per cent perchloric acid in 10 minutes at 100°, was considerably advanced, with only slight color development from 1.0 mg per cc of most other sugars. In hydrochloric acid, most other sugars in these quantities developed considerable color with tryptophane in 10 minutes at 100°.

*Technique and Specificity of Reaction*—Maximum color development for amounts of desoxyribose of 0.15 mg per cc required 2 mg of tryptophane or a molar ratio of tryptophane to desoxyribose of approximately 9:1. A minimal final perchloric acid concentration necessary for rapid stable color development was 30 per cent. The method employed in the estimation of DNA in purified nucleic acid fractions or nucleoproteins was as follows. To 1.0 cc aliquots of solution were added 0.2 cc of 1 per cent *DL*-tryptophane in 0.01 N sodium hydroxide, and 1.2 cc of 60 per cent perchloric acid, and the mixture was heated in a vigorously boiling water bath for 10 minutes. The colored solution was rapidly cooled to room temperature and read within 5 minutes in a Klett-Summerson photoelectric colorimeter, the filter of which had a transmission range of 485 to 550 m $\mu$ .

Under these conditions, sodium desoxyribonucleate solutions of 0.1 to 0.5 mg per cc yielded a linear relationship between concentration and color intensity, and the readings of duplicate estimations were a maximum of 2.5 per cent apart. The reading due to 0.1 mg of DNA per cc was over 40, indicating that the method could be used readily in the range of 0.05 to 0.5 mg per cc of desoxyribonucleate. Two impediments to direct colorimetry of the reaction mixture containing nucleoprotein were the incomplete solution of colorless protein particles and the development of colors due to protein constituents. The first was eliminated by filtration of the turbid solution through hardened paper. In the second case, isoamyl alcohol (b.p. 132°) extracted the colored product of desoxyribose-tryptophane condensation without extracting various other materials. These alcoholic extracts may then be employed in colorimetry at room temperature, after clarification by centrifugation at 2000 R.P.M. for 10 minutes. By means of this variation of the method, a reproducible linear relationship was also obtained when 2.0 cc of isoamyl alcohol were used to extract, by vigorous mixing, 2.4 cc of reaction mixture. Perchloric acid exhibits a considerable solubility in isoamyl alcohol and thus increased



the volume of the extract depending on the volume of extractant employed. Extraction of the alcoholic phase with water or alkali discharged the color, although the solution was still strongly acid. Addition of perchloric acid did not cause reappearance of the color before heating.

The validity of these procedures may be established by the demonstration that the absorption spectrum of the colored solution derived from an unknown mixture is identical with that of known DNA, or that the ratios of color intensities in the photoelectric colorimeter with various filters are the same for DNA and the unknown, at least within the range of 460 to 560 m $\mu$ . Since it was observed that thymus nucleohistone developed a color in this reaction at a rate slightly less than did DNA, although total color production over extended heating periods was directly proportional to the desoxyribose content of both substances, it was considered desirable to employ the former as a standard in the estimation of protein bound nucleic acid. Free DNA was used in the estimation of the DNA of purified nucleic acid fractions. The desoxyribose content of these materials was considered to be 4.32 times the phosphorus content, 4.32 being the ratio of molecular weights of desoxyribose to phosphorus. The necessity of standardizing these materials by their phosphorus content is stressed.

In Table I, the reactions produced by various substances in the perchloric acid-tryptophane reaction under the conditions described above are compared with those of the diphenylamine reaction, carried out according to Dische (6). The data summarized in this table indicate that materials producing a color in one reaction will generally produce a color in the other reaction under the conditions given. Interfering reactions are markedly given by aldehydes, fructose, and agar. The former reaction is much more sensitive to small amounts of fructose and derivatives.

*Absorption Spectra*—2 cc of various carbohydrate solutions were heated with 0.4 cc of 1 per cent tryptophane and 2.4 cc of 60 per cent perchloric acid in a boiling water bath for periods necessary to develop the predominant colored product. After cooling, they were extracted with 4.0 cc of isoamyl alcohol. The alcohol was removed with a pipette, clarified by centrifugation, and examined over the range of the visible spectrum at room temperature in a Beckman spectrophotometer. Some of the observed absorption spectra are presented in Fig. 1.

The spectra resulting from the reactions of DNA and desoxyguanosine were identical, indicating that the reaction was due to the carbohydrate common to both materials rather than an unusual base in the former. As will be demonstrated later, the color intensity was exactly proportional to the desoxyribose contents of these compounds. The dissimilarities of the spectra due to DNA and fructose do not permit a simple optical separation of these materials by the use of an appropriate filter. The

similarities of the colored products of ribose and furfural with tryptophane indicated that furfural was produced on heating ribose with perchloric acid. The decomposition of xylose appeared to proceed somewhat differently.

TABLE I  
*Colorimeter Readings of Carbohydrates in Desoxyribose Reactions*

Substance		Perchloric acid tryptophane			Diphenylamine		
		Filter 420	Filter 520	Filter 660	Filter 420	Filter 540	Filter 660
	mg per cc						
Sodium desoxyribonucleate	0 10	21	52	0	39	61	32
Ribose	1 0	42	22	14	37	22	33
Xylose	1 0	22	7	1	11	5	13
Arabinose	1 0	9	8	7	8	6	11
Glucose	1 0	19	12	7	14	2	11
Glucose 1 phosphate*	1 0	7	9	5	12	3	9
Fructose	0 10	39	35	10	22	15	21
Fructose 6 phosphate*	1 0	234	160	43	74	29	62
Fructose-1,6 diphosphate*	1 0	177	117	34	44	16	23
Mannose	1 0	14	6	0	3	6	7
Galactose	1 0	23	13	3	6	5	12
Glucosamine	1 0	13	9	7	9	4	8
Rhamnose	1 0	19	23	4	5	0	0
Inositol	1 0	3	2	2	4	1	8
Ascorbic acid	1 0	240	119	26	37	15	0
Furfural	0 05	64	26	12	33	21	28
Acetaldehyde	0 20	64	47	15	39	19	24
Benzaldehyde	0 65	18	16	11	22	8	11
Palmitaldehyde glyceryl acetal	1 0	4	0	4	36	18	12
Glyceraldehyde	1 0	580	96	35	660	500	168
Heparin	1 0	8	1	2	34	29	13
Hyaluronic acid	1 0	9	3	1	2	5	0
Chondroitin sulfate*	1 0	10	4	5	39	38	22
Agar	0 50	195	92	28	78	56	136
Starch	1 0	1	0	0	0	1	0
Gum arabic	1 0	30	12	7	0	1	0

\* Potassium salts

*Estimation of Total Desoxyribose*—It has been noted that the diphenylamine reaction enabled the estimation of purine desoxyriboside exclusively in DNA (1), yielding a ratio of purine-bound desoxyribose to total desoxyribose of exactly 1/2. This has been confirmed in this laboratory for the DNA isolated from thymus nucleohistone. An examination of desoxyguanosine, DNA, and thymus nucleohistone, by means of the perchloric

acid-tryptophane reaction, revealed that this reaction enables the estimation of pyrimidine desoxyriboside, the desoxyribose of which is liberated at a rate markedly different from that of purine-bound desoxyribose. The hydrolysis of the pyrimidine-desoxyribose linkage is apparently facilitated by the substitution of the 4-5 double bond of the pyrimidine nucleus (12). It is likely that perchloric acid functions in a manner similar to other peracids in the conversion of ethylenic compounds into glycols. Thus, above  $80^{\circ}$ , the use of perchloric acid enabled the hydrolysis of pyrim

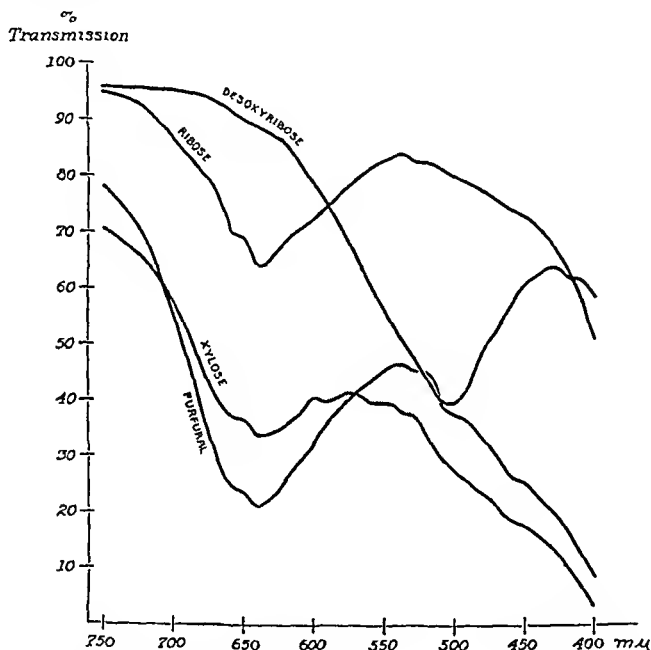


FIG 1 Absorption spectra of condensation products of desoxyribose and other aldehydes with tryptophane

idine nucleosides and color development from this type of bound desoxyribose. The manner in which the color development of desoxyguanosine DNA, and thymus nucleohistone solutions varied with the temperature and the time of heating is presented in Fig 2.

A desoxyguanosine solution of 0.100 mg per cc, a sodium desoxyribonucleate solution of 0.0120 mg of phosphorus per cc, and a thymus nucleohistone solution of the same phosphorus content were prepared. These concentrations are theoretically equivalent to a total free desoxyribose content of 0.0518 mg per cc. The ratios of color intensity produced by these substances in the diphenylamine reaction were 2:1:1.

The following were placed in 50 cc cylinders fitted with ground glass stoppers 7.0 cc of each solution or water, 1.4 cc of 1 per cent tryptophane in dilute sodium hydroxide, and 8.4 cc of 60 per cent perchloric acid. The contents were mixed thoroughly and the cylinders were immersed in an oil bath at 80°, 90°, 100°, and 115°. At suitable intervals, the cylinders were inverted several times and aliquots were removed. The solutions were placed in an ice bath for 1 minute, at room temperature for 1 minute, and read in the colorimeter. At 100° and 115°, brown tinges appeared in the red solution after extended periods of heating. Extraction of the red

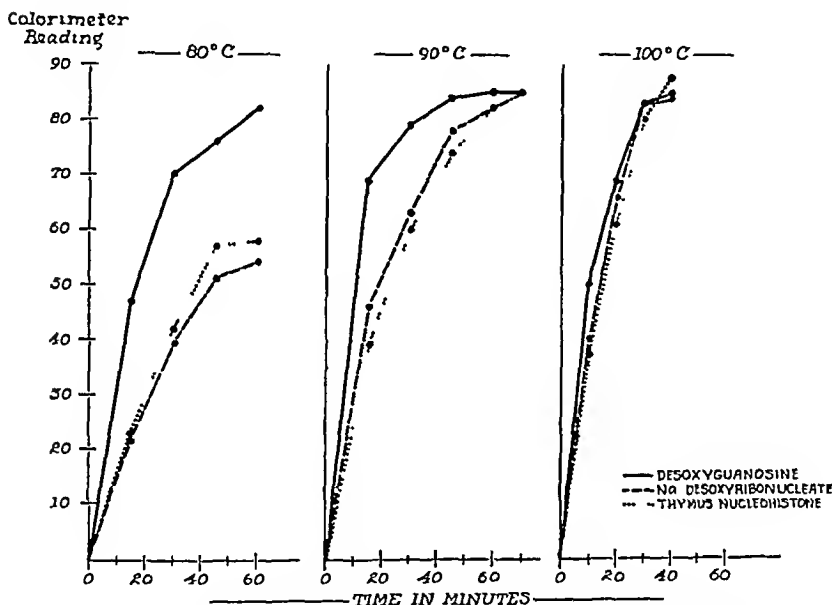


FIG. 2. Rate of color development at different temperatures of various desoxyribose derivatives.

product with isoamyl alcohol left the brown pigment in the aqueous phase, the colorimetric reading of which, after correction for volume change, could be subtracted from the total colorimeter reading. These corrected values for the colors produced in the reaction mixtures demonstrated that color development, at times past those presented in Fig. 2, was essentially complete. Since the ratios of color intensities for the three substances at 70 minutes and 40 minutes at 90° and 100° respectively were 1:1:1, and the rates of color increase were negligible, it was concluded that the total desoxyribose of nucleic acid reacted under these conditions.

*Estimation of Total Ribose*—Similar experiments were performed to as-

certain whether pyrimidine ribosides were also hydrolyzable. Since, in this case, the color development depended on a conversion of ribose to furfural, the percentage of perchloric acid used in the reaction mixture was increased to reduce the time of reaction. The substrates employed in these tests were *d*-ribose, guanosine, and ribonucleic acid at concentrations of 0.100 mg of ribose per cc. The reaction mixtures were heated at 100°, 110°, and 120°, the volume of 1 per cent tryptophane being one fifth that of the ribose solution, and the ratios of volumes of 60 per cent perchloric acid to ribose plus tryptophane being 1.0, 1.5, and 2.0. At suitable intervals, the walls of the stoppered cylinders were washed with the reaction mixture, and 1 cc aliquots were removed and added to 2 cc of isoamyl alcohol. The mixture was cooled in running water and the green alcoholic supernatant was examined in the colorimeter, the filter of which had a transmission range of 640 to 700 m $\mu$ . In view of the observed absorption spectrum, a filter of slightly lower transmission range was perhaps more desirable. The aqueous phase contained brown side reaction products. After a 60 minute interval at 120° in 1.5 volumes of 60 per cent perchloric acid, the hydrolysis and conversion to furfural of purine and pyrimidine ribosides in ribonucleic acid proceeded at a rate enabling the estimation of total ribose, with ribose or guanosine as a standard. That is, the ratio of color intensities of ribose to guanosine to ribonucleic acid was 1:1:1 under these conditions.

*$\beta$ -Indole Derivatives in Desoxyribose Reaction*— $\beta$ -Methyl indole, indole-3-acetic acid, and indole-3-propionic acid were tested under the same conditions and molar concentrations in which tryptophane was used. The color intensities due to the reaction of the indole derivative with 30 per cent perchloric acid alone were in the order: tryptophane < indole-3-acetic acid < indole-3-propionic acid <  $\beta$ -methyl indole. The first three of these produced red condensates with desoxyribose in perchloric acid, that due to indole-3-acetic acid being markedly less than the other two. The absorption spectra in the visible range of the alcoholic extracts of the reaction products were determined and that due to tryptophane was the simplest. It appeared from these studies that tryptophane was the most satisfactory, readily available  $\beta$ -indole derivative for use in the estimation of desoxyribose.

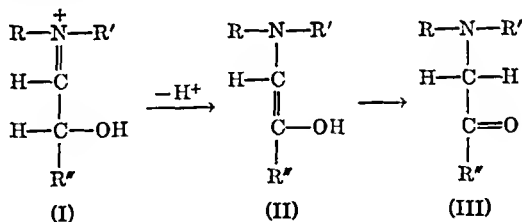
#### DISCUSSION

Perchloric acid has proved to be a most useful reagent in the hydrolysis of all types of nucleosides and in the condensation of desoxyribose and other aldehydes with tryptophane. The comparative stability of sugars in this strong acid as contrasted to their behavior in concentrated HCl and H<sub>2</sub>SO<sub>4</sub>, as indicated by the slow rate of formation of colored reaction

products with tryptophane, is to be noted. Studies on tryptophane in mixtures of acetic anhydride and perchloric acid indicate that  $\text{HClO}_4$  facilitates the substitution of indole N (13). Use has been made of this acid as a catalyst in the acetylation of sugars (14) and in the formation of acetals (15).

The color reactions of the carbohydrates have been empirically valuable in the identification and estimation of some sugars. The nature of the various reaction products has not been ascertained in most cases, nor have hypotheses as to reaction mechanisms been proposed. A hypothesis to describe adequately the phenomena observed in the perchloric acid-tryptophane reaction must account for (1) the specificity of the reaction of desoxyribose as contrasted to ribose or glucose, (2) the shift in absorption maximum from the desoxyribose to furfural condensates, (3) the rôle of tryptophane, and (4) the rôle of the acid. Various reactions of the nitrogen-glycosides have recently proved subject to generalization in the light of the Amadori reaction (16). It is considered likely that the specificity of the reaction of desoxyribose with secondary amines to yield the colored products observed is a function of the inability of 2-desoxy sugars and some aldehydes to participate in the Amadori reaction.

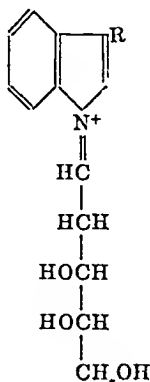
In the Amadori reaction, the cation of the Schiff base of a nitrogen-glycoside becomes rearranged as shown in formulas (I) to (III).



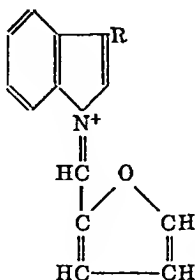
It is apparent that in this system the condensation of 2-hydroxy carbohydrates with tryptophane will not increase the number of conjugated double bonds in proceeding to the ketone (III). However, 2-desoxyribose and some other aldehydes will be unable to proceed past (II) and will exist in equilibrium with (I), the structures of which are given in formulas (IV) to (VI). Thus desoxyribose, furfural, and benzaldehyde yield products (IV), (V), and (VI), which are red, green, and blue respectively, and contain one, three, and four additional double bonds, conjugated to the indole nucleus.

The perchlorates of various secondary amines such as diphenylamine and indole derivatives are colorless (17). The similarity of colors produced in the presence of  $\text{HCl}$  also attests to the non-auxochromic character

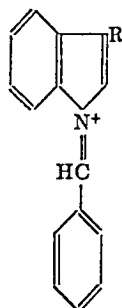
of perchlorate ion in the production of the colored derivative. It appeared that hydrolysis of the glycoside was promoted by dilution of the acid.



(IV)



(V)



(VI)

The direction of shift of the absorption maxima is in agreement with the increase in conjugated double bonds of the postulated derivatives and thus is in support of the hypothesis. Glyceraldehyde yielded a yellow color in this reaction, and this would also be in accord with the general mechanism, if the assumption is made that an additional double bond was formed at the 2-3 linkage, as in the decomposition of glycerol to acrolein or in some other position of conjugation, to yield a total of two additional double bonds.

Although the colors produced in the diphenylamine reaction at 100° do not fit this hypothesis, the same reaction at room temperature over extended periods produced colors with these compounds which possess a similar shift in the proper direction. Thus desoxyribose gave a reddish purple, furfural a green, and benzaldehyde a blue color. This suggests that the heating procedure in the diphenylamine reaction as commonly carried out results in some more complex course of events. This fact, which did not agree with the proposed mechanism, suggested that it was desirable to reexamine the diphenylamine reaction at a lower temperature, with the above mentioned results.

Since it appears possible to predict, as in the diphenylamine reaction under mild conditions, the color produced as a function of the increase in resonating potential of the product of condensation of aldehyde and base, the choice of a quantitative colorimetric reaction for either of these substances may be somewhat assisted. A knowledge of the structures and reactivities in various condensing agents of the aldehydes or secondary amines to be estimated, and the behavior of the impurities likely to be

present, enable the development of a specific colored product for purposes of estimation

Many naturally occurring substances interfere to a greater or lesser degree with both reactions. In the perchloric acid-tryptophane method, aldehydes, fructose, and its derivatives are among the most important of these interfering substances. Both reactions have been effectively used on purified nucleic acid and nucleoprotein preparations, yielding complementary results. However, it is necessary to emphasize that the estimation of desoxyribose nucleic acid in biological products by either reaction should employ optical controls concerning the nature of the color produced. The elimination of interfering impurities must be undertaken prior to the application of the reactions directly to the crude mixtures. It may be expected that different biological systems will require different procedures of estimation of this acid.

The author is indebted to Miss V R Brown for competent technical assistance. He is grateful to Sharp and Dohme, Inc., and to Mr J Ciminera of that company, for the use of the Beckman spectrophotometer. He wishes to acknowledge indebtedness to Dr S Gurrin and Dr O Meyerhof of the Department of Biochemistry of the University of Pennsylvania, and to Dr A Mirsky of the Rockefeller Institute, for preparations of some of the difficultly obtainable carbohydrates and their derivatives. Some of these materials were prepared in the laboratory of the late Dr P A Levene, who had thus prepared the material, as well as theoretical, basis for the continuation of research on the nucleic acids.

#### SUMMARY

The use of perchloric acid and tryptophane in the estimation of desoxyribose has been described. Conditions for the estimation in some substances of both purine and pyrimidine nucleosides of desoxyribose and ribose have been presented. The reactivities of numerous carbohydrates have been compared in the perchloric acid-tryptophane and diphenylamine reactions. A mechanism for the former reaction has been proposed and its implications have been discussed.

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# INVESTIGATIONS OF AMINO ACIDS, PEPTIDES, AND PROTEINS

## XVIII THE AMINO ACID REQUIREMENTS OF *LEUCONOSTOC MESAENTEROIDES* P-60\*

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It was reported by Snell, Strong, and Peterson (2) in 1937 that *Leuconostoc mesenteroides* P-60 grew and produced acid when incubated at 28° for 5 to 6 days with a culture medium containing acid-hydrolyzed peptone, glucose, sodium acetate, tryptophane, cystine, six inorganic salts, riboflavin, and any one of the supplements of an alcohol-soluble liver fraction, Difco yeast extract, and ground pork liver. After subsequent determination by Snell and coworkers that pantothenic acid (3, 4), tryptophane (5), and purines (6, 7), but not riboflavin (8, 9), are essential nutrients for this microorganism, the original basal medium was modified by substituting acid-hydrolyzed casein for peptone and adding adenine, guanine, uracil, thiamine, pantothenic acid, pyridoxine, biotin, and folic acid (10, 11). Bohonos, Hutchings, and Peterson (12) have shown that pyridoxine stimulates the growth of *Leuconostoc mesenteroides* P-60, while Gaines and Stahley (13) have determined the growth requirements of *Leuconostoc mesenteroides* Strain 535.

An investigation of the amino acid requirements of *Leuconostoc mesenteroides* P-60 was undertaken by the present authors in order that, if possible, microbiological assay procedures might be devised for the determination of amino acids in proteins in addition to those for which comparable methods are available. From preliminary investigations of *Streptococcus lactis* R, *Lactobacillus pentosus*, *Streptococcus salivarius*, and *Leuconostoc mesenteroides* P-60 it was considered that the last microorganism was the most promising for amino acid assay purposes.

If a given microbiological procedure is to be satisfactory for the quantitative determination of an amino acid (or other nutrient) in biological materials, the amino acid must be essential for the growth of the microorganism, the blank titration (or turbidity) value should be low, the basal

\* For Paper XVII in this series see Dunn *et al* (1)

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medium should contain the minimal concentration of nutrients required to produce a standard curve of maximal slope, the standard curve should be reproducible within close limits and approximately linear over the segment to be employed for analytical purposes, and the assay should be applicable over a 4-fold, and preferably greater, range of concentration

Experience has shown that these criteria must be met if the stimulatory or inhibitory influence of amino acids or other substances present in protein hydrolysates or other amino acid-containing solutions is to be avoided. Although there is wide variation in the precision and accuracy with which particular amino acids may be determined by microbiological procedures, the most favorable conditions are high specificity of the microorganism for the amino acid and relatively high proportion of the amino acid in the solution subjected to analysis. The latter factor may become relatively unimportant if the experimental solution may be diluted sufficiently to minimize the effect of any interfering substances. It has been observed, also, that maximal precision and accuracy result when the lower part of the standard curve is employed for assay purposes.

It appears that most investigations of the nutritional requirements of microorganisms have been empirical and limited rather than systematic and complete, since a prohibitive length of time would be required to determine the environmental conditions and the types, proportions, and levels of nutrients which are optimal for the growth of a microorganism. It should be possible, however, to investigate basal media for use in the quantitative determination of amino acids in protein materials by a logical plan with a minimum of arbitrary assumptions.

A basal medium essentially of the same composition as that employed by Snell (5), except that amino acids were substituted for casein hydrolysate, was adopted arbitrarily in the present work. Acid production was measured at a series of levels of each essential amino acid, a standard curve for each amino acid was constructed from the titration data, and the minimal quantity of each amino acid required to give the maximal titration was estimated from its standard curve. The next step was to determine the minimal level of the whole group of amino acids at which the slope of the standard curve for a particular amino acid (lysine) was maximal<sup>1</sup>. A new medium was prepared of the same composition as the original except that each essential amino acid was introduced at the minimal level found to be optimal, acid production was measured as before, and a new standard curve for each amino acid was prepared. If the minimal amounts of an amino acid required to give the maximal titration did not differ sig-

<sup>1</sup> Multiple medium experiments have been employed to a limited extent in investigations of media for the assay of pantothenic acid (14, 15) with *Lactobacillus casei* and amino acids (16) with *Lactobacillus arabinosus*.

nificantly in the two experiments, it was assumed that the indicated proportion of the amino acid was optimal. If necessary, this experimental procedure was repeated until a constant minimal level of each amino acid resulted. Finally, the composition of the near optimal basal medium was determined from these data.

#### EXPERIMENTAL

The assay technique was that described in previous publications from the authors' laboratory. The microorganism, *Leuconostoc mesenteroides* P-60, was obtained from the University of Texas through the courtesy of Dr. E. E. Snell. The culture was carried on yeast-dextrose agar (Difco). Subcultures were made at weekly intervals, were incubated<sup>2</sup> for 24 hours at 37°, and were stored in the refrigerator. The inoculum was grown on the basal medium in which 50 mg of hydrochloric acid-hydrolyzed casein supplemented with cystine and tryptophane were substituted for the amino acids and ammonium chloride. The inoculum was grown for 24 hours in 15 ml centrifuge tubes and, after centrifugation, was suspended in 10 ml of sterile saline. 1 drop of the suspension was used to inoculate each tube. Experiments were run in duplicate or quadruplicate with 5 ml of the basal medium (made up to twice the concentration shown in Table I) diluted finally to 10 ml. The tubes were autoclaved 10 minutes at 15 pounds pressure and incubated 72 hours at 35–37°. The titrations were made with standard base with biomothymol blue indicator.

Curves showing the experimental results are given in Figs. 1 to 22, a

#### DISCUSSION

Consideration of the experimental results (Figs. 1 to 21) obtained with the original basal medium (Table I, Medium A) led to the conclusion that seventeen of the twenty-one amino acids were essential for the growth of *Leuconostoc mesenteroides* P-60 under the stipulated experimental conditions. It appeared that alanine, hydroxyproline, norleucine, and norvaline were non-essential or auxiliary growth substances<sup>3</sup>. From the appearance of the standard curves for arginine, cystine, glutamic acid, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, tryptophane,

<sup>2</sup> The optimal temperature for sauerkraut fermentation has been shown to be 18.3° to 21.1° (17) and it may be that incubation of *Leuconostoc mesenteroides* at this temperature would be an advantage in bioassays with this microorganism.

<sup>3</sup> If significant amounts of any one of these amino acids were present as impurities in the basal medium, this conclusion would be invalid. That one (presumably hydroxyproline) and possibly more amino acids are contaminated with proline appears probable since the blank titrations in the multiple medium experiments performed with proline increased from about 0.7 ml of 0.05 N NaOH to about 11 ml over a 5-fold range of levels of amino acid in the basal medium.

TABLE I  
Composition of Basal Media

Constituent	Medium A	Medium B	Medium C	Medium D
	mg per l	mg per l	mg per l	mg per l
<i>dl</i> -Alanine	200	400	3000	2000
<i>l</i> (+)-Arginine HCl	200	100	120	80
Asparagine (natural)*	200	400	600	400
<i>l</i> (-) Cystine	200	100	80	120
<i>l</i> (+) Glutamic acid*	200	200	200	150
Glycine	200	100	160	100
<i>l</i> (-) Histidine HCl H <sub>2</sub> O	200	200	20	20
<i>l</i> (-)-H <sub>3</sub> droxyproline†	200	50	80	100‡
<i>dl</i> -Isoleucine	200	200	120	150
<i>l</i> (-)-Leucine*	200	100	80	75
<i>dl</i> -Lysine HCl*	200	400	240	160
<i>dl</i> -Methionine	200	100	60	40
<i>dl</i> -Norleucine	200	50	80	100‡
<i>dl</i> -Norvaline	0	50	80	100‡
<i>dl</i> -Phenylalanine	200	200	120	60
<i>l</i> (-)-Proline†	200	100	80	25
<i>dl</i> Serine	200	200	160	80
<i>dl</i> -Threonine†	200	200	400	450
<i>l</i> (-)-Tryptophane	50	50	20	10
<i>l</i> (-) Tyrosine	200	50	40	30
<i>dl</i> Valine	200	200	120	150
Glucose	10 (gm )	20 (gm )		
Adenine sulfate	12	12		
Guanine hydrochloride	12	12		
Uracil	12	12		
Sodium acetate	6 (gm )	12 (gm )		
NH <sub>4</sub> Cl	3 "	6 "		
KH <sub>2</sub> PO <sub>4</sub>	500	500		
K <sub>2</sub> HPO <sub>4</sub>	500	500		
MgSO <sub>4</sub> 7H <sub>2</sub> O	200	200		
NaCl	10	10		
FeSO <sub>4</sub> 6H <sub>2</sub> O	10	10		
MnSO <sub>4</sub> 4H <sub>2</sub> O	10	10		
Thiamine hydrochloride§	2	1		
Pyridoxine§	2	1 6		
<i>dl</i> -Calcium pantothenate§	0 1	2		
Riboflavin§	0 2	2		
Nicotinic acid§	0 2	2		
Biotin	1 5 (γ)	5 (γ)		
Folic acid ¶	1 0 "	2 "		
<i>p</i> Aminobenzoic acid**	0 1 "	0 1 (γ)		

Same as for Medium B

Same as for Medium B

TABLE I—*Concluded*

\* The natural and *dl* forms were employed interchangeably, the latter at twice the concentration of the former

† Merck and Company, all other amino acids were Amino Acid Manufactures' products

‡ Employed at this level in each of the five multiple medium experiments

§ Gelatin Products Company

|| Merck and Company, crystalline biotin

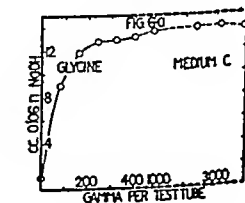
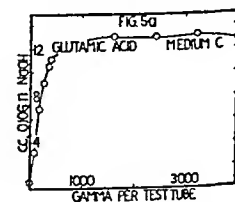
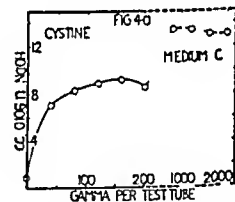
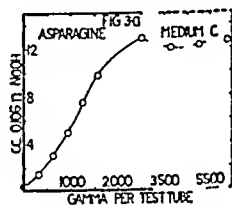
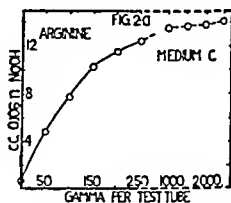
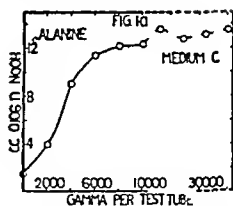
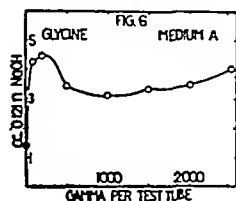
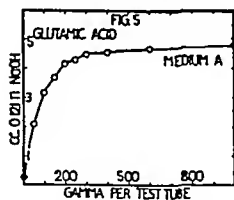
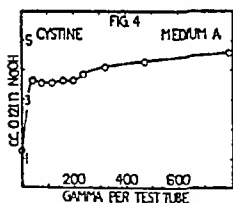
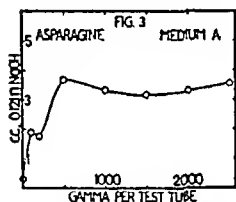
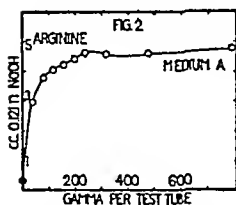
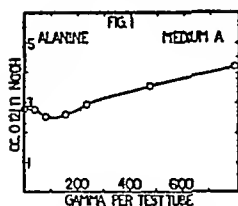
¶ Prepared from The Wilson Laboratories' liver powder 1:20 by norit adsorption and elution according to the procedure of Hutchings *et al* (18). This solution containing 0.77  $\gamma$  of folic acid per ml was used only in Medium A. The folic acid used in Media B, C, and D was obtained from the University of Texas through the courtesy of Dr. H. K. Mitchell. The 0.5 mg sample containing 30,000 units of folic acid was dissolved in 250 ml of hot water. The solution was diluted to 500 ml with 95 per cent ethanol and stored in the refrigerator.

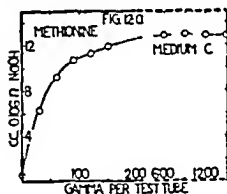
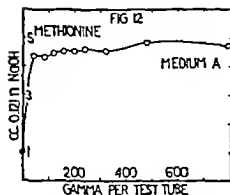
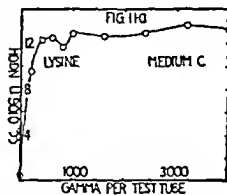
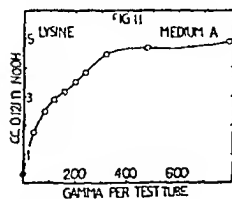
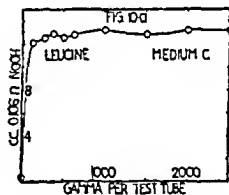
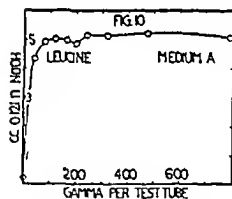
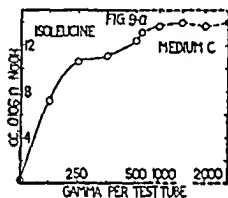
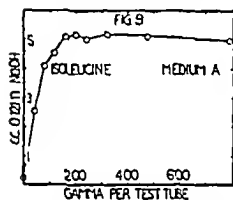
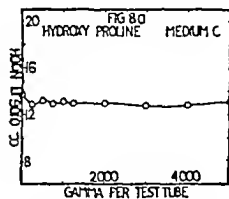
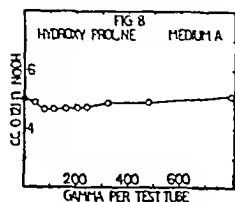
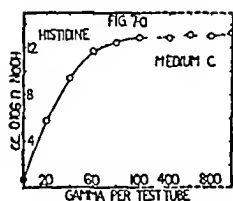
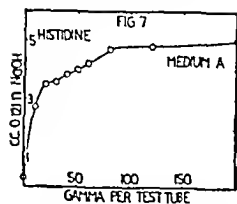
\*\* Eastman Kodak Company

tyrosine, and valine it seemed probable that satisfactory procedures could be devised for the quantitative determination of these amino acids. It seemed desirable, however, to investigate further the nutritional requirements of the microorganism in order that, if possible, the inhibitions in the standard curves for serine, glycine, and other of the amino acids might be eliminated. It was desired, also, to increase acid production<sup>4</sup> and to extend the limits of the linear segments of many of the curves.

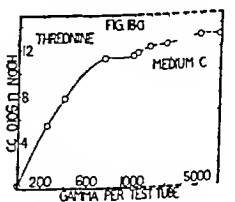
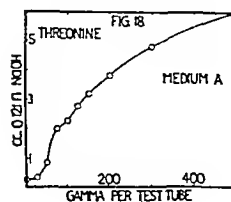
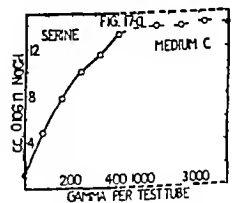
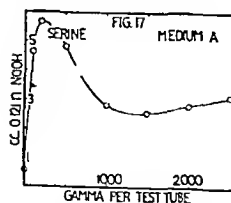
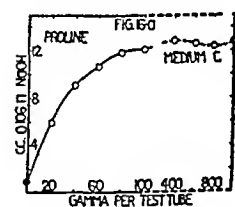
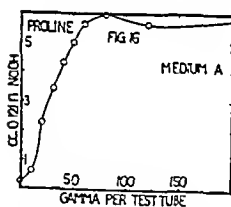
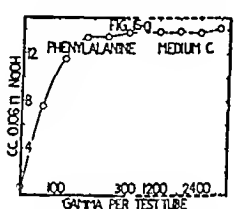
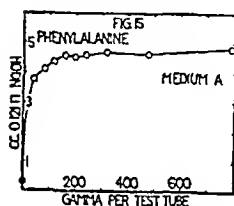
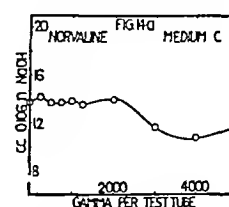
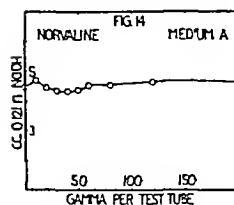
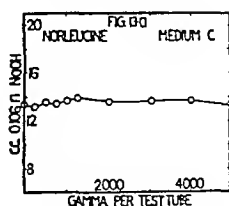
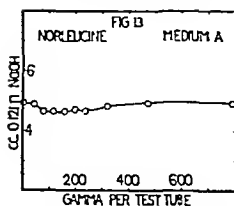
The composition of Medium A was first changed by doubling the concentration of glucose, sodium acetate, and ammonium chloride. The results which seemed most significant were elimination of inhibition in the asparagine curve and an increase in total acid production with increasing levels of asparagine, lysine, and histidine. Marked alterations of the amino acid concentrations were made in keeping with the results of the preliminary experiments and Medium B of the composition shown in Table I was prepared and tested. Since smooth standard curves were obtained for nearly all of the amino acids and acid production was further increased, it seemed evident that Medium B was superior to Medium A. Medium C of an amino acid composition, corresponding to the maxima of the standard curves obtained with Medium B, was prepared and tested with the results shown in Figs. 1, a to 21, a. Acid production and alanine requirement were markedly increased and the initial linear segments of the standard curves were extended from an average of about 50  $\gamma$  to about 140  $\gamma$  per tube. Medium C was tested further over an 8-fold range of levels of total

<sup>4</sup> Since insufficient acid for convenient titration is produced by *Leuconostoc mesenteroides* grown on culture media commonly used for this purpose, McMahan and Snell (19) have suggested that the growth response of this microorganism be measured turbidimetrically.

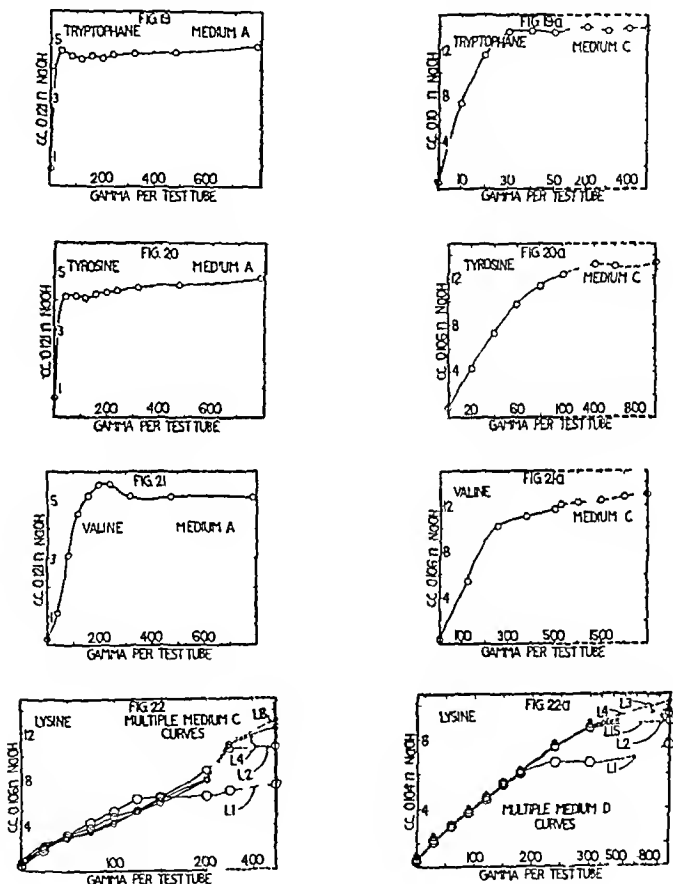








amino acids at a series of levels of one of the amino acids (lysine). Although the standard curves (Fig 22) were not greatly different over this range, acid production at the 4-fold level was maximal and depressions appeared in the initial segments of the lysine standard curves. It seemed



desirable, therefore, to redetermine the maximum of each amino acid standard curve at the 4-fold level of total amino acids. Medium D was prepared in keeping with these data and was tested at five levels over a 4-fold range of total amino acids at a series of levels of one amino acid (lysine).

From the results of this experiment (Fig 22, a) it was concluded that the initial level of total amino acids probably could be employed for assay purposes up to about 180  $\gamma$  of *dl*-lysine monohydrochloride per tube. On the other hand, the other curves were nearly superimposable at all levels of *dl*-lysine and it appeared that such standard curves might be used for the assay of lysine up to quantities of about 240  $\gamma$  of *dl*-lysine monohydrochloride per tube.

It is apparent that a basal medium whose composition is determined as described is not necessarily truly optimal and that other media containing widely varying types, proportions, and levels of amino acids as well as other essential nutrients may be satisfactory for quantitative assay purposes. It may be pointed out, also, that further improvements in the basal medium might be effected by investigating the proportions and levels of other constituents of the medium at the level of the amino acids found to be near optimal. The results of some experiments designed to measure the salt effect may illustrate this point. The observation that high salt concentrations significantly inhibit the growth of *Leuconostoc mesenteroides* P-60 under the observed conditions is of particular importance in connection with assays of amino acids in acid hydrolysates of proteins, since relatively high concentrations of salt would be present after such solutions have been brought to pH 7 by the addition of base. In such cases the bulk of the acid should be removed prior to the neutralization of the solution. On the other hand it has been found that nearly constant acid production occurs at each level of lysine over a range of about 20 to 120  $\gamma$  in solutions containing from about 0.7 to 2 per cent of sodium chloride. Media most satisfactory for bioassay purposes should contain about 0.7 per cent salt<sup>5</sup> in order that inhibitions which occur at lower changing concentrations of salt may be avoided and that an unknown solution containing concentrations of salt as high as possible may be introduced with minimal alteration in the response of the microorganism.

#### SUMMARY

Amino acids essential for the growth of *Leuconostoc mesenteroides* P 60 in a medium of known but arbitrarily selected composition have been determined. A systematic although not complete study has been made of the acid produced by this microorganism with varying proportions and at different levels of amino acids. It has been found that *Leuconostoc mesenteroides* P-60 appeared to grow satisfactorily in the final improved medium, and standard curves considered to be satisfactory for amino

<sup>5</sup> According to Prescott and Dunn (17) a salt concentration of 2.5 per cent is favored by commercial sauerkraut manufacturers.

acid bioassay purposes were obtained. It has been concluded, tentatively, that it may be possible to determine a number of amino acids in protein hydrolysates with the aid of this microorganism and the described basal medium.

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# INVESTIGATIONS OF AMINO ACIDS, PEPTIDES, AND PROTEINS

## IX THE DETERMINATION OF LYSINE IN PROTEIN HYDROLYSATES BY A MICROBIOLOGICAL METHOD\*

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(Received for publication, September 8, 1944)

Microbiological methods<sup>1</sup> for the determination of arginine, isoleucine, glutamic acid, tryptophane, and valine in protein hydrolysates with *Lactobacillus arabinosus* and *Lactobacillus casei* have been described by the workers quoted in an earlier paper from the authors' laboratory (1). An

\* For Paper XVIII in this series see Dunn *et al.* (6).

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<sup>1</sup> A microbiological method for the determination of leucine in proteins with a (leucineless) mutant strain of *Neurospora crassa* has been described recently by Ryan and Brand (2). The values for leucine in gelatin, egg albumin, and horse hemoglobin were in substantial agreement with those obtained in other laboratories from analyses of the identical preparations by the solubility product and the isotope dilution methods. The experimental evidence presented is considered to support the conclusion of these authors concerning the reliability of their microbiological method and one can agree entirely with their recommendation that we should "refrain from considering microbiological values as significant without other confirmation." On the other hand the criticism of Ryan and Brand that assays of amino acids with *Lactobacillus arabinosus* and *Lactobacillus casei* which have been reported are lacking in dependability does not appear to be entirely justified. While it is correct that percentages of leucine and valine found by Hegsted (3) in casein were appreciably lower than the values reported by McMahan and Snell (4) and Kuiken *et al.* (5), figures in good agreement with those given by the last workers have been obtained by the present authors (1). It seems probable, therefore, that some essential nutrients were lacking or were present in suboptimal levels in the basal medium which Hegsted employed. The opinion has been advanced by Ryan and Brand that the accuracy of the value for isoleucine in casein reported by Kuiken *et al.* (5) may be in doubt because the value for isoleucine in gelatin reported by the latter workers did not agree with that calculated by subtracting the percentage of leucine in gelatin determined by the *Neurospora crassa* procedure from the total leucine-isoleucine in gelatin determined chromatographically or by Dakin's method. On the other hand, the view that the percentage of isoleucine in gelatin reported by Kuiken *et al.* may be approximately correct is strengthened by the observation that the percentages of leucine and isoleucine in casein found by the present authors (1) are in close agreement with values reported by Kuiken *et al.* On the whole the percentages of amino acids in proteins determined with the *Lactobacilli* procedures, developed almost simultaneously and

analogous procedure for the determination of lysine in protein hydrolysates with *Leuconostoc mesenteroides* P-60 is described in the present paper

#### EXPERIMENTAL

The assay technique and other experimental details were the same as those given in previous publications from the authors' laboratory. The casein, the silk fibroin, and the hydrolysis procedure were the same as those described in an earlier paper (1) and the improved basal medium (Table I, Medium D) is given in the preceding paper (6). The eight standard curves for lysine which were obtained, each from a duplicate assay, closely resembled each other and the standard curves for lysine shown in the preceding paper (6). The experimental data are given in Tables I to V.

#### DISCUSSION

It may be concluded from the results of the assays shown in the tables that lysine may be determined with reasonable accuracy with the aid of *Leuconostoc mesenteroides* P-60 and the authors' improved basal medium. Additional support for this view was derived from experiments in which lysine was recovered from the casein hydrolysate by a method analogous to that employed previously with *l*(+)-glutamic acid (1). A mixture containing about 8  $\gamma$  of *l*(+)-lysine per ml was prepared by adding 4.00  $\gamma$  per ml of *l*(+)-lysine (as the monohydrochloride) to an aliquot of the casein hydrolysate. The mixture was assayed for lysine by the described microbiological procedure. In a series of six experiments the mean deviations from the mean of the values at the different levels were 3.0, 4.7, 6.1, 4.5, 8.1, and 7.0 per cent with an average value of 5.6 per cent. The percentages of lysine recovered in these experiments were 108, 106, 116, 103, 100, and 101 per cent with an average value of 106 per cent.

It may be pointed out that the intrinsic errors inherent in the assay of lysine in a recovery experiment may lead to recovery data which may be either much less or greatly more accurate than is indicated by the calculated percentages. This uncertainty arises because the errors of the assay

investigated extensively in a number of laboratories (1), appear to agree as closely with each other and with the most reliable literature data as do the values for leucine reported by Ryan and Brand. While only leucine, biotin, sugar, and salts are needed for the growth of the *leucineless* strain of *Neurospora crassa* in contrast to the multiple and interdependent growth factors required by the *Lactobacilli*, there are some obvious disadvantages inherent in the use of a fungus for bioassay purposes. A different mutant strain is required for the determination of each amino acid, relatively few assays can be completed in a given time, and the mutant may undergo "a partial adaptation to the wild type condition." According to Ryan and Brand about one-third of the values obtained in the assay of horse hemoglobin and excluded in calculating the average value for leucine were ascribed to adaptive changes undergone by the mutant.

of the amino acid in the hydrolysate and the recovery mixture enter into the calculation and may be additive or cancel each other. If the average recovery were much beyond  $100 \pm 10$  per cent, one would not be inclined to place confidence in the reliability of the assay procedure. On the other hand the apparent error in a recovery of  $100 \pm 10$  per cent may be as much as 3 times the actual error in an assay of a mixture prepared by mixing aliquots of the hydrolysate and a solution of the amino acid, each containing

TABLE I  
*Results of Assay of Lysine in Casein Hydrolysate*

Casein per tube	Titration volume of 0.104 N NaOH per tube	Lysine* found	
		Per tube	Per ml sample
$\gamma$	ml	$\gamma$	$\gamma$
497	3.35	40.7	40.7
497	3.30	37.5	37.5
994	5.14	78.1	39.1
994	5.02	75.6	37.8
1491	6.94	116.0	38.7
1491	6.52	117.1	39.0
1988	8.49	148.1	37.0
1988	8.44	147.4	36.9
2480	10.56	191.8	38.4
2480	9.33	165.9	33.2

\* An average of 37.8  $\gamma$  of lysine was found per ml of casein hydrolysate sample. The average mean deviation from the mean of the values at the different levels was 3.6 per cent. The percentage of lysine in the casein uncorrected for moisture and ash was calculated to be 7.60. It was found from comparable assays of three additional aliquots of the same casein hydrolysate that the lysine in casein uncorrected for moisture and ash was 7.93, 7.60, 7.56, and 7.75 per cent in the four samples with an average value of 7.64 per cent. The mean deviations from the mean of the values at the different levels were 5.6, 3.6, 3.1, and 4.1 per cent with an average value of 4.1 per cent. The following percentages of lysine in casein uncorrected for moisture and ash were found in the fifteen assays which were performed: 6.93, \*7.14, \*7.90, 7.35, 7.94, 7.36, 7.45, 7.75, 8.02, 7.93, 7.60, 7.56, 7.75, and 9.00. \* It is of interest that the average of these values is 7.72 per cent and that the average figure is 7.71 per cent if the least reliable starred values are excluded.

the same amount of the test amino acid. For analogous reasons recovery percentages with low apparent errors are not necessarily a true indication of the accuracy of the assay.

It is evident that recovery experiments with test mixtures of amino acids of a composition simulating that of the protein under investigation are of value in determining the possible deleterious influence exerted by amino acids in the protein hydrolysate on the assay value of a particular



amino acid It is true, however, that the errors introduced by hydrolytic products other than amino acids are not measured in an experiment of this

TABLE II

*Results of Assay of Lysine in Amino Acid Test Mixtures*

Test Mixture 1				Test Mixture 2†			
Amino acid mixture per tube	Titration volume of 0.104 N NaOH per tube	Lysine‡ found		Amino acid mixture per tube	Titration volume of 0.104 N NaOH per tube	Lysine§ found	
		Per tube	Per ml sample			Per tube	Per ml sample
$\gamma$	ml	$\gamma$	$\gamma$	$\gamma$	ml	$\gamma$	$\gamma$
863	3.30	36.8	36.8	420	2.56	20.0	20.0
863	3.30	36.8	36.8	420	2.66	21.2	21.2
1726	4.76	67.0	33.5	840	4.10	45.5	22.7
1726	4.75	66.9	33.5	840	4.08	45.0	22.5
2589	6.38	101.3	33.8	1260	5.29	64.5	21.5
2589	6.80	110.0	36.7	1260	5.10	68.0	22.6
3452	7.97	136.8	34.2	1680	6.01	82.0	20.5
3452	8.62	152.2	38.0	1680	5.90	80.0	20.0
4315	9.30	168.9	33.8	2100	7.00	100.8	20.2
4315	9.86	183.5	36.7	2100	7.22	105.0	21.0

\* The composition of the test mixture simulating casein was the same as that given in a previous paper (1) except that the mixture contained 4.29 per cent of *l*(+) lysine.

† Test Mixture 2 contained 100 mg per 100 ml of each of the amino acids listed in Test Mixture 1.

‡ An average of 35.4  $\gamma$  of lysine was found per ml of amino acid test mixture sample. The average mean deviation from the mean of the values at the different levels was 4.6 per cent. The percentage of lysine recovered in the assay was 95.6 per cent. In a series of eight analogous experiments the mean deviations from the mean of the values at the different levels were 4.6, 5.7, 6.3, 5.6, 3.4, 4.6, 3.7, and 4.4 per cent with an average value of 4.8 per cent. The percentages of lysine recovered in these experiments were 95.6, 97.5, 92.1, 106.2, 109, 105.4, 100.5, and 105.4 per cent with an average value of 101.5 per cent.

§ An average of 21.2  $\gamma$  of lysine was found per ml of amino acid test mixture sample containing 4.76 per cent *l*(+) lysine. The average mean deviation from the mean of the values at the different levels was 4.6 per cent. In a series of sixteen analogous experiments with test mixtures containing the percentages of *l*(+) lysine given in parentheses, the mean deviations from the mean of the values at the different levels were 4.3 (33.3), 10.1 (4.8), 7.4 (33.3), 6.3 (4.8), 2.9 (33.3), 6.4 (4.8), 4.8 (33.3), 4.6 (4.8), 2.3 (33.3), 3.8 (4.8), 2.8 (0.50), 5.3 (0.50), 3.8 (0.50), 4.4 (0.50), 7.0 (0.50), and 4.6 (0.50). The percentages of lysine recovered in these experiments listed in the same order and corresponding to the foregoing data were 87.7, 96.9, 97.0, 98.5, 106.5, 94.5, 100.0, 106.0, 111.0, 109.5, 101.4, 99.2, 101.5, 101.0, 114.0, and 95.5 per cent with an average value of 100.6 per cent.

type even in the case of purified proteins whose amino acid composition is known most completely. At the present time both types of recovery ex

periments are performed in the authors' laboratory in order that as much data as possible will be available for the evaluation of procedures and amino acid results. While it is possible in the case of most purified proteins to assess the dependability of a given microbiological procedure and a particular assay value by comparing the latter with data reported in the literature it is not possible at the present time to judge the probable accuracy of

TABLE III

*Composition of Amino Acid Test Mixtures Simulating That of Silk Fibroin\**

Amino acid	Test Mixture 3		Test Mixture 4	
		Per cent free base natural form		Per cent free base amino acid
	mg		mg	
<i>dl</i> -Alanine	1056	16.9	528	23.9
<i>l</i> (+)-Arginine HCl	18.4	0.15	18.4	0.22
<i>l</i> (+)-Glutamic acid	40.0	1.25	40.0	1.51
Glycine	876	28.0	876	39.6
<i>l</i> (-)-Histidine HCl H <sub>2</sub> O	1.89	0.045	1.89	0.086
<i>dl</i> -Isoleucine	50.0	0.80	25.0	1.13
<i>l</i> (-)-Leucine	25.0	0.80	25.0	1.13
<i>l</i> (+)-Lysine HCl	6.25	0.16	29.5†	0.54†
<i>dl</i> -Methionine	103.6	1.66	51.8	2.31
<i>dl</i> -Phenylalanine	60.0	0.96	30.0	1.36
<i>l</i> (-)-Proline	20.0	0.64	20.0	0.91
<i>dl</i> -Serine	544	8.7	272	12.3
<i>dl</i> -Threonine	61.6	0.99	30.8	1.29
<i>l</i> (-)-Tyrosine	264	8.5	264	11.9

\* The percentages of the fourteen amino acids in silk fibroin listed by Colbr Edsall (7) were employed to determine the composition of the amino acid mixture. Glutamic acid was included in approximately the proportion found by the present authors (1). According to unpublished experiments of the present authors, silk fibroin contains several per cent of valine and it seems probable that it also contains aspartic acid, cystine, hydroxyproline, tryptophane, and possibly other amino acids. The quantities of amino acids listed above were dissolved in water and the solution diluted to 100 ml. with 3 N HCl.

† Calculated as free base *l*(+) lysine.

evidence reported in the literature. In an analysis of Merck's (Hammarsten) casein containing 13.75 per cent nitrogen, Albanese (8) found that the lysine nitrogen was 10.22 per cent of the total nitrogen. This value is equivalent to 8.2 per cent of lysine in casein containing 15.4 per cent nitrogen. In the procedure employed by Albanese, arginine and histidine were removed from the catholyte of the electrodialysate as the flavianate and  $\text{HgCl}_2$  complexes, respectively, and lysine was calculated from the total nitrogen of the final filtrate. While it was shown that the procedure was

TABLE IV  
*Results of Assay of Lysine in Silk Fibroin Hydrolysate*

Silk fibroin per tube	Titration volume of 0.104 N NaOH per tube	Lysine* found	
		Per tube	Per ml sample
$\gamma$	ml	$\gamma$	$\gamma$
8,050	3.65	47.0	47.0
8,050	3.70	48.0	48.0
16,100	5.49	85.7	42.9
16,100	5.50	85.8	42.9
24,150	9.11	161.1	53.7
24,150	8.88	156.6	52.2
32,200	11.02	202.2	50.6
32,200	11.37	212.0	53.0
40,250	11.60	219.0	43.8
40,250	12.78	272.0	54.4

\* An average of 48.9  $\gamma$  of lysine was found per ml of silk fibroin hydrolysate. The average mean deviation from the mean of the values at the different levels was 8.0 per cent. In a series of ten analogous experiments the mean deviations from the mean of the values at the different levels were 8.7, 8.0, 6.3, 7.7, 9.1, 9.2, 4.6, 6.4, 4.1, and 5.0 per cent. The percentages of lysine in silk fibroin uncorrected for moisture and ash and listed in order corresponding to the foregoing data were 0.61, 0.61, 0.58, 0.56, 0.62, 0.49, 0.47, 0.64, 0.52, and 0.49 per cent with an average value of 0.56 per cent. It has been found by calculations made after the described investigations were completed that the italicized percentages of lysine may have resulted from excessive salt in the hydrolysate samples analyzed. In view of this finding the average uncorrected per cent of lysine in silk fibroin may be somewhat higher than 0.56 per cent.

satisfactorily precise when applied to mixtures of the pure bases and solubility corrections were made in calculating the lysine value, it is evident that any nitrogenous impurities in the final lysine-containing filtrate would be determined as lysine.

In 1913 Van Slyke (9) determined the lysine in Merck's (Hammarsten) casein by his nitrogen distribution procedure. The lysine nitrogen found, 10.3 per cent of the total nitrogen, is equivalent to 8.3 per cent of lysine in casein containing 15.4 per cent nitrogen. While it is generally con-

sidered that the nitrogen distribution method is subject to error, particularly when applied to crude proteinogenous materials (10, 11), Van Slyke (9) and Gortner and Sandstrom (12) have shown that lysine may be recovered with relatively high accuracy from mixtures of pure amino acids. Van Slyke (9) has also reported data of relatively high precision in the analysis of lysine in the hydrolysates of casein and other proteins. The

TABLE V  
*Results of Assay of Lysine in Amino Acid Test Mixture 4\**

Amino acid mixture per tube	Titration volume of 0.05 N NaOH per tube	Lysine found	
		Per tube	Per ml sample
$\gamma$	ml	$\gamma$	$\gamma$
1383	1.75	7.6	15.2
1383	1.83	8.3	16.6
2766	2.60	15.1	15.1
2766	2.72	16.2	16.2
4149	3.18	21.5	14.3
4149	3.20	21.8	14.5
5532	3.70	28.0	14.0
5532	3.65	27.8	13.9
6915	4.15	34.5	13.8
6915	4.35	37.2	14.9

\* An average of 14.8  $\gamma$  of lysine was found per ml of amino acid test mixture sample containing 0.53 per cent of l(+) lysine. The average deviation from the mean of the values at the different levels was 5.0 per cent. In a series of three analogous experiments with test mixtures containing the percentages of l(+) lysine given in parentheses the mean deviations from the mean of the values at the different levels were 5.0 (0.53), 6.4 (0.53), and 4.0 (0.53) per cent and the percentages of lysine recovered in these experiments were 100, 102, and 93 per cent with an average value of 98 per cent. In comparable experiments with amino acid Test Mixture 3 recoveries of lysine found were 68, 75, and 79 per cent of the amounts of l(+) lysine added. These values are only approximate because of the marked inhibition which occurred and it is considered that this test mixture is unsatisfactory probably because of the relatively low proportion of lysine and the relatively high proportion of certain other amino acids.

It has been found by calculations made after the described investigations were completed that the foregoing low percentages of lysine may have resulted from excessive salt in the samples analyzed.

lower values<sup>2</sup> (5.0 to 7.9 per cent of lysine in casein) reported by other workers (14-18) are probably less dependable than the figure obtained by Van Slyke. It is of interest that the percentage of lysine derived from nitrogen distribution data would tend to be lowered, rather than raised, by the majority of the probable errors inherent in the method.

<sup>2</sup> Plummer and Lowndes (13) found 9.38 per cent of lysine in casein.

The specific decarboxylase procedure of Neuberger and Sanger (19) has been employed recently in modified form by Zittle<sup>3</sup> for the determination of lysine in casein. It was found that Pfanstiehl's casein contained  $7.8 \pm 0.3$  per cent lysine calculated on the ash- and moisture-free basis.

Lysine in protein hydrolysates has been most commonly determined as its picrate after precipitation of lysine as its phosphotungstate from the filtrate obtained by precipitating arginine and histidine as their silver salts from the hydrolysate. This method, devised by Kossel and his associates (20-22), was employed in 1901 by Hart (23) who found 5.8 per cent of lysine in casein calculated on the basis of 15.9 per cent nitrogen in casein found earlier by Chittenden. Since that date values ranging from 5.65 to 7.0 have been reported by various workers<sup>4</sup> (18, 25-29) although the value, 6.25, found in 1933 by Vickery and White (30) has been most commonly accepted. In a more recent publication Vickery (31) has expressed some doubt concerning the accuracy of his figure for lysine as well as concerning the reliability of the Kossel procedure as modified in his laboratory. This opinion seems to be supported by the observations that the value 7.61 per cent for lysine in casein was reported by Osborne and Mendel (32) in 1914 and the values 6.99 and 7.5 per cent<sup>5</sup> were reported by Van Slyke (9) in the same year. The lysine picrate which served as the basis for the latter figure was shown to be analytically pure by amino nitrogen analysis.

It appears probable from the experimental evidence in the literature and that presented in this paper that purified casein contains approximately 8 per cent of lysine. While it is not inconceivable that all of the closely agreeing values obtained by the described electrodialysis, nitrogen distribution, specific decarboxylase, and microbiological procedures may have been erroneously high, the fact that 7.5 per cent, the figure found by isolating the analytically pure picrate, is necessarily the minimal value gives some credence to the view that the true figure may be approximately 8.0 per cent. To what extent the source of the milk used for the preparation of casein, the method by which casein is prepared, the purity of the casein,

<sup>3</sup> Private communication from Dr. Charles A. Zittle, Parke, Davis and Company, Detroit, Michigan. Dr. Zittle has pointed out in a further communication received after the present paper was in galley proof that the procedure followed by Neuberger and Sanger was suggested by the work of Gale and Epps (36) in whose laboratory bacterial decarboxylases have been studied for many years. It is of interest, also, that Neuberger and Sanger found that ash- and water free casein contained 6.7 per cent lysine.

<sup>4</sup> Calvery (24) reported that the lysine nitrogen of casein is 6.48 (average of eight values ranging from 6.07 to 6.77) per cent of the total nitrogen. The percentage of lysine in casein equivalent to this value cannot be calculated from the data given.

<sup>5</sup> Calculated by the present authors from the values given for the lysine nitrogen in terms of per cent of total nitrogen of the casein samples.

the method employed in the hydrolysis of the casein, and other factors may lead to deviations in the lysine percentage has not been determined

The lysine content of silk fibroin was found to be 0.56 per cent, uncorrected, and 0.60 per cent corrected for the 5.68 per cent moisture and 0.25 per cent ash reported previously (1). In a series of recovery experiments the percentages of lysine recovered were 66, 69 per cent at relatively high levels of the recovery mixture, and 107 and 103 per cent at relatively low levels. The latter data may be more reliable because at relatively high dilutions of the hydrolysate sample inhibitions from amino acids and salt are minimized. It is considered that, because of the unusually high content of alanine, glycine, serine, and tyrosine and the low content of lysine in silk fibroin, the bioassay of lysine in the hydrolysate of this protein presents a severe test of the microbiological procedure.

Fischer and Skita (33) reported in 1902 that the lysine content of silk fibroin was too low to determine by Kossel's method but Abderhalden (34) found 0.85 per cent and Vickery and Block (35) found 0.25 per cent of lysine in this protein by the Kossel procedure. Apparently, no other values are reported in the literature.

#### SUMMARY

A method has been described for the quantitative determination of l(+)-lysine in protein hydrolysates with *Leuconostoc mesenteroides* P-60. Assay data which are considered to be of satisfactory precision and accuracy have been presented. Casein and silk fibroin were found to contain 8.3 and 0.6 per cent, respectively, of lysine.

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# STUDIES ON THE HEMORRHAGIC SWEET CLOVER DISEASE

## XIV HYPERPROTHROMBINEMIA INDUCED BY METHYLYXANTHINES AND ITS EFFECT ON THE ACTION OF 3,3'-METHYLENEBIS (4-HYDROXYCOUMARIN)\*

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Several observations have been recorded which suggest that the methylxanthines have an effect on blood coagulation. Klemperer in his treatise, "Untersuchungen ueber Gicht," published in 1896, expressed the thought that caffeine evoked the development of a coagulative ferment ((1) p. 59). In 1920 Nonnenbruch and Szyzka suggested that the methylxanthines caused an increase in fibrin ferment (2). Subsequently Meissner (3) and Addicks (4) reported that administration of theophylline shortened the coagulation time of whole blood. The authoritative review by Morawitz on blood and blood diseases (5) and the monograph by Pickering ((6) p. 213) cite the use of a preparation composed of theophylline and ethylenediamine in equal proportions as a hemostatic agent. More recently Tobitani (7) reported that substances containing the guanidine nucleus decrease the blood clotting time by increasing the formation of thrombin, while Sirasaka (8) has indicated that the administration of caffeine to rabbits shortened the bleeding time.

The purpose of this report is 2-fold: first to show that the methylxanthines, caffeine (1,3,7-trimethylxanthine), theobromine (3,7-dimethylxanthine), and theophylline (1,3-dimethylxanthine), induce in the dog, rat, and rabbit a state of hyperprothrombinemia as reflected by shortened plasma prothrombin times, secondly, to indicate that, as a result of the induced hyperprothrombinemia, the hypoprothrombinemic action of the anticoagulant 3,3'-methylenebis(4-hydroxycoumarin) is lessened (9).<sup>1</sup>

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<sup>1</sup> The basic observations recorded here have been known to us since May, 1942. In March, 1943, a collation of the findings was circulated privately among certain



### Methods

Previous publications give all the basic techniques employed as well as the rationale of handling the animals. Specific citations are made when necessary. In this study, as in all the previous work (10-12), whole plasma (100 per cent) and the 50, 25, 12.5, and 6.25 per cent plasma concentrations were routinely explored for prothrombin level (or activity), but since the change in prothrombin activity reflected by the 12.5 per cent plasma (1 part plasma, 7 parts saline solution) is readily detected and reproducible, most of the data are given only in terms of this value.

It should be emphasized that a marked prolongation of the prothrombin time (hypoprothrombinemic state) is possible with the anticoagulant 3,3'-methylenebis(4-hydroxycoumarin) ((11) pp 950-953). In contrast, the extent to which the prothrombin time can be shortened from the normal by the methylxanthines (hyperprothrombinemic state) is limited. Although the reduction in seconds from the normal is of a small order, the decrease is readily reproducible. Prothrombin concentration varies hyperbolically with shift in prothrombin time in the particular time range in which the hyperprothrombinemia is measured. Hence a *large* increase in prothrombin concentration may produce only a *small* change in prothrombin time (see (10) Fig 1 and pp 8-10).

The status of our knowledge on prothrombin activity (or concentration) as reflected by readings in seconds does not permit of a direct evaluation of *decreased* as opposed to *increased* prothrombin times. A decrease of 2 to 4 seconds from the normal prothrombin time of 12.5 per cent plasma might conceivably involve an actual change in prothrombin concentration (or activity) equivalent to that which occurs when the time is prolonged 20 to 40 seconds above the normal.

### EXPERIMENTAL

#### *Hyperprothrombinemic Action of Methylxanthines*

*Conditions of the Experiments*—The dogs were maintained on a diet of skim milk powder 40, yellow corn 15, meat scraps 15, wheat bran 10, wheat middlings 10, alfalfa meal 7, bone meal 2, and salt 1. They were not fasted either before or during the test period. The rats were handled exactly as previously (12), which includes a 12 hour fast before the methylxanthine was given, the rabbits as in (10, 11), except that they were not fasted.

#### *Effect of Single Oral Dose of Theobromine on Plasma Prothrombin Time—*

clinical investigators studying the action of 3,3'-methylenebis(4-hydroxycoumarin) in man. The first public report was made for us on February 6, 1944, by Dr. Shepard Shapiro in the seminar on blood coagulation that he conducted in the Department of Therapeutics, New York University.

Results from many trials will be reported in a highly condensed form. A representative response for dog plasma is given in the dilution curves of Fig 1 and the curve labeled "Theobromine" of Fig 2. Curve A of Fig 1 gives the prothrombin times of the normal plasma, Curve B of plasma from the same dog 48 hours after feeding 200 mg per kilo of theobromine. The shortened prothrombin times over the whole range of the

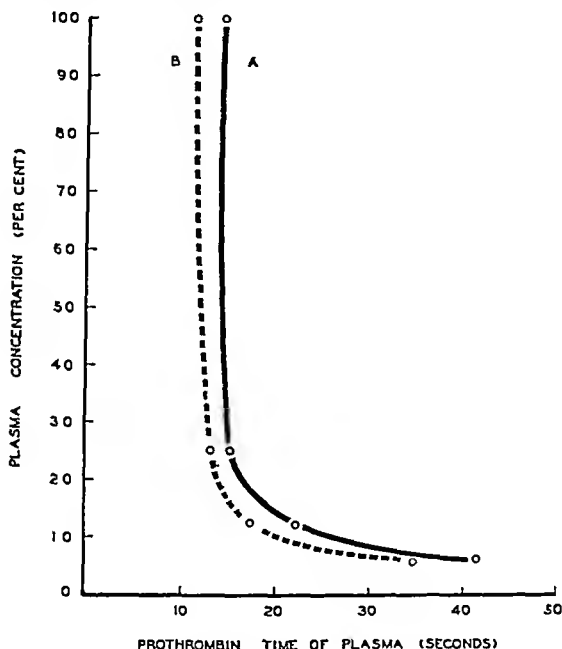


FIG 1 The effect of theobromine on the prothrombin time of dog plasma. Curve A represents the normal plasma, Curve B, plasma from the same dog 48 hours after feeding theobromine (200 mg per kilo)

dilution curve are apparent. Comparable responses are realized when caffeine or theophylline is given.<sup>2</sup>

All three methylxanthines evoke shortened prothrombin times of the same general order when given orally to dogs, rabbits, and rats (Table I)

<sup>2</sup>Single doses of the following preparations used clinically were also tested: Aminophylline (theophylline with ethylenediamine), theocin (theophylline with sodium acetate), diuretin (theobromine with sodium salicylate), caffeine citrate (caffeine with citric acid). The response evoked by these combinations, based in the main on trials with dogs which need not be given in detail, was substantially the same as that produced by the methylxanthine itself.

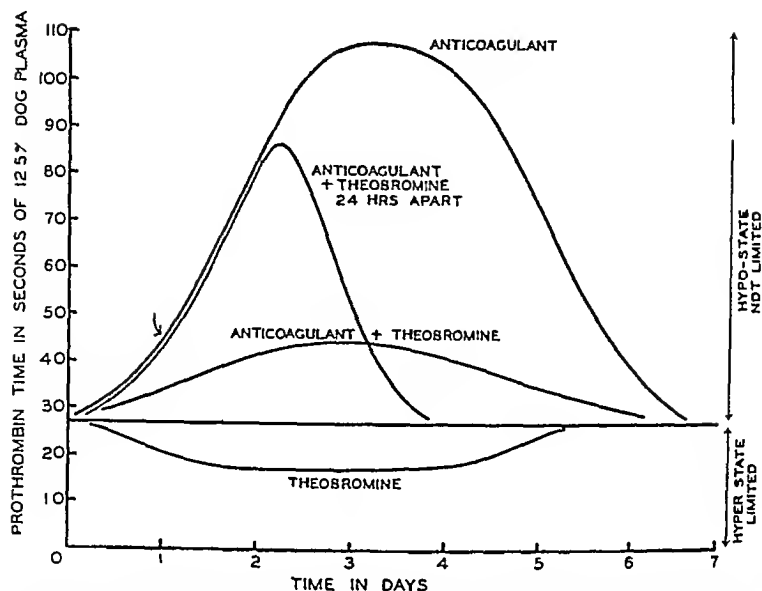


FIG 2 Representative chart of the protective action of theobromine against 3,3'-methylenebis(4-hydroxycoumarin) in the dog. Theobromine, 100 mg per kilo, and 3,3'-methylenebis(4-hydroxycoumarin) 10 mg per kilo, given orally

TABLE I

*Representative Effect of Caffeine on Prothrombin Time of 12.5 Per Cent Plasma of Various Species\**

Species	Caffeine fed†  mg per kg	Prothrombin time in sec of 12.5 per cent plasma						
		Normal prothrombin time	12 hrs after feeding	24 hrs after feeding	48 hrs after feeding	72 hrs after feeding	144 hrs after feeding	168 hrs after feeding
Dog	50	30.4 ± 0.6‡	28.8	29.0	30.2			
	100	29.0 ± 1.0	27.5	28.0	25.2	25.7	28.0	
	200	22.5 ± 0.4	17.0	18.0	17.2	16.0	23.5	
Rat	4	40.0 ± 0.8	38.2	35.4				
	100	37.9 ± 0.5	35.8	35.0	34.5	38.2		
Rabbit								
	100	30.2 ± 0.4	27.0	22.0	21.7	24.1	23.4	28.0

\* Each line of figures is a series of values obtained with a single animal

† As caffeine citrate

‡ Standard deviation values

The degree of the response varies in extent and duration, depending on the dose (Table II). Individual variation between members of the same

species is detectable. There is also a variation in response between species. In sum the detectable dose under our conditions in a 250 to 300 gm rat is 1 to 2 mg, in the rabbit 10 to 25 mg per kilo, in the dog 25 to 50 mg per kilo.

*Effect of Repeated Doses of Theophylline on Prothrombin Time of 12.5 Per Cent Dog Plasma*—Aminophylline (theophylline-ethylenediamine) was fed three times daily to dogs at the 4, 8, and 12 mg per kilo level. This dosage level is equivalent to the range recommended in Osler-Christian for clinical practice (14). The hyperprothrombinemic effect usually became detectable in 2 to 5 days at the levels indicated. Individual dogs have been maintained in the hyperprothrombinemic state with these levels.

TABLE II

*Effect of Different Dosage Levels of Theophylline on Prothrombin Time of 12.5 Per Cent Dog Plasma\**

Theophylline fed†	Prothrombin time in sec. of 12.5 per cent						
	Normal prothrombin time	12 hrs after feeding	24 hrs after feeding	48 hrs after feeding	72 hrs after feeding	96 hrs after feeding	120 hrs after feeding
mg per kg							
10	30.8 ± 0.6‡	30.0	30.0	29.6	30.6		
50	32.0 ± 0.7	27.2	27.4	25.0	33.0	32.1	
75	27.0 ± 1.2	26.9	22.2	23.8	24.5	27.2	28.4
200	30.0 ± 1.3	29.0	27.0	26.6	25.8	23.4	29.4
400	31.2 ± 0.5	22.6	22.6	25.3	24.8	23.8	31.8

\* Each line of figures represents a series of values obtained with a single dog.

† As theocine (theophylline and sodium acetate).

‡ Standard deviation values based on not less than four and usually more than ten values, obtained over a period of 2 to 3 months with the same dog. Dogs maintain relatively constant prothrombin times over prolonged periods under ordinary conditions (13).

for periods up to 30 days. During the first 2 weeks the intensity of the response was relatively uniform. There followed then a period with detectable fluctuations. A typical protocol of a dog receiving 12 mg per kilo of aminophylline thrice daily is given in Table III.

*Action of Other Purines, Pyrimidines, and Related Substances*—In the dog and rabbit, xanthine (2,6-dioxypurine) in doses up to 200 mg per kilo was found to be devoid of any hyperprothrombinemic action. Eight out of a group of twenty-two rats showed a border line response at the 100 mg level. Heteroxanthine<sup>3</sup> (7-methylxanthine) showed slight activity in the rat at 100 mg. None of the following showed activity in doses of

<sup>3</sup> Synthesized for us here by Mr. Saul Roseman.

200 mg per kilo in either the rat, dog, or rabbit, uric acid, adenine, allantoin, urea, creatine, creatinine, guanidine, glycoyammine, arginine, uracil, 6-methyluracil, 2-methyl-5-ethoxymethyl-6-oxypyrimidine<sup>4</sup>

*Evidence That Hyperprothrombinemic Effect of Active Methylxanthines Is Not Due to Hemoconcentration*—Since diuresis is one of the effects that may be evoked by theophylline and caffeine, the urine output of the dogs was measured under the conditions of the tests. The 24 hour average excretion during a 3 to 5 day period, prior to the administration of the methylxanthine, was first ascertained. The drug was then fed at the levels at which it produced a readily detected hyperprothrombinemia. No increase in the pre-test urine excretion was noted. This is in agreement with previously recorded observations that the methylxanthines are usually

TABLE III  
*Effect of Repeated Doses of Theophylline*

	Prothrombin time of 12.5 per cent plasma
	sec
Normal value	35 0
1st day after giving aminophylline	30 2
2nd " " " "	28 7
4th " " " "	30 2
6th " " " "	31 5
12th " " " "	31 0
3 days after aminophylline was withdrawn	33 5
6 " " " " "	34 8 (Normal)

not effective as diuretics in normal non-edematous animals ((15) p 638). It has also been indicated that the main diuretic effect of theophylline is usually dissipated within a few hours after administration (16). Furthermore, it has been recorded that theophylline, rather than causing dehydration of the blood stream, actually produces a slight rise in plasma volume (17).

For control purposes the following pertinent determinations were made on dog blood at the plateau of the hyperprothrombinemic response, total plasma protein (18), total plasma nitrogen (19), hemoglobin, and hematocrit (20). No increase in any of these constituents was noted.

As additional evidence that the hyperprothrombinemic effect is not due to diuresis, the action of ammonium chloride, sodium acetate, and

<sup>4</sup>The last two compounds were kindly supplied by Dr R T Major, Research Department, Merck and Company, Inc, Rahway, New Jersey. Professor Henry Borsook, California Institute of Technology, Pasadena, supplied the glycoyammine

urea was studied. While these substances produced a measurable diuresis, only an occasional and transitory hyperprothrombinemia was noticeable 12 hours after administration. Colossal doses of these substances (1 gm per kilo of urea and 800 mg per kilo of the inorganic salts) did not induce a consistent or prolonged hyperprothrombinemia. In contrast the methylxanthines do so when the dose is relatively small.

*Evidence That Hyperprothrombinemia Induced by Methylxanthines Is Not Due to Vitamin K-Like Action*—The methylxanthines were tested for antihemorrhagic potency (vitamin K activity) by the procedure of Almquist and Klose (21). 1 day-old chicks were fed a vitamin K-deficient diet, to which the methylxanthines had been added at the level of 10 and 100 mg per kilo of ration. The clotting time of the whole blood, as well as the prothrombin time of the chick plasma, was determined after 1, 2, and 3 weeks. While the addition of 10 mg per kilo of 2-methyl-1,4-naphthoquinone (menadione, Abbott) to the ration reduced the clotting time of whole blood to 3 to 4 minutes, the whole blood clotting time of the chicks receiving the methylxanthines remained at 20 minutes. Since the assay for antihemorrhagic potency is subject to wide variations, we asked Dr. Carl Nielsen and Mrs. F. P. Dann of the Abbott Laboratories, North Chicago, Illinois, to make control tests for us by the method that they use routinely (22). They also realized negative results with the methylxanthines. Topelberg and Honorio (23) have reported that caffeine does not decrease the prolonged clotting time of chicks deprived of vitamin K.

*Effect of Methylxanthines on Action of 3,3'-Methylenebis(4-hydroxycoumarin)*

*Effect of Methylxanthines on Action of 3,3'-Methylenebis(4-hydroxycoumarin) in the Dog*—The individual response of 30 dogs of varying age, weight, and breed to the action of the anticoagulant was first ascertained. The detectable dose of the anticoagulant in the dogs used in these trials under the conditions of the standardization (no fasting), is approximately 2.5 mg per kilo. The actual dose of the anticoagulant used varied from 7.5 to 15 mg per kilo. For each dog an individual response curve comparable to that given in ((11) Fig 3, p 945) was constructed with whole plasma as well as plasma of various dilutions. But the presentation of the data will be restricted to the 10 mg per kilo dose of the anticoagulant. It should be emphasized that at this dosage level 3,3'-methylenebis(4-hydroxycoumarin) is capable of inducing a marked hypoprothrombinemia, representing a prolongation of the 12.5 per cent prothrombin time from the average normal value of 25 seconds to 100 to 110 seconds in 48 to 72 hours. Normal prothrombin times were usually restored in from 5 to 7 days.

The dose of methylxanthine varied from 10 to 200 mg per kilo. Representative responses of individual dogs to the simultaneous administration of anticoagulant and methylxanthines<sup>5</sup> are given in Table IV and Fig 2, curve labeled "Anticoagulant + theobromine."

*Duration of Protective Effect of Methylxanthines against Action of 5,5'-Methylenebis(4-hydroxycoumarin) in the Dog*—The individual hypoprothrombinemic response evoked by a standard dose of the anticoagulant in mature dogs, rabbits, and rats is quite uniform on a given diet (10-12). It was therefore surprising to find that after a methylxanthine is administered to dogs simultaneously with the anticoagulant, the response to repeated standard doses of the anticoagulant (10 mg per kilo) is altered. A rep-

TABLE IV

*Representative Effects of Methylxanthines on Action of 5,5'-Methylenebis(4-hydroxycoumarin) in the Dog*

Dose of methylxanthine per kilo	Prothrombin time in sec. of 12.5 per cent plasma			
	After 48 hrs		After 96 hrs	
	10 mg per kilo anticoagulant (control)	10 mg anticoagulant* + methylxanthine	10 mg anticoagulant (control)	10 mg anticoagulant + methylxanthine
25 mg theophylline	64	28	53	26
200 " "	76	60	99	22
10 " theobromine	102	54	84	36
100 " "	89	31	106	35
10 " caffeine	47	31	67	33
100 " "	95	34	61	25

\* The average normal 12.5 per cent prothrombin time is 25 seconds, range 22 to 29 seconds.

resentative example of the capacity of theobromine to impart a sustained resistance in the dog is illustrated in Table V.

The duration of this unique protective effect is dependent on the dose of methylxanthine originally administered. In dogs receiving 50 or 100 mg per kilo of either caffeine, theobromine, or theophylline, the resistance persisted for approximately 14 weeks, at the 10 to 25 mg per kilo level, 6 to 8 weeks. Xanthine, at the 100 mg per kilo level, gave a slight protection, but the resistance usually disappeared within 3 weeks.

*Effect of Methylxanthines on Action of 5,5'-Methylenebis(4-hydroxycoumarin) When Given after Anticoagulant in the Dog*—When methylxanthines are given to dogs in the process of developing the hypoprothrombinemic

<sup>5</sup> A border line protective action by xanthine was noted at the 100 mg per kilo level. Allantoin, uric acid, urea, and guanidine showed only a slight but variable protective action at levels up to 100 mg per kilo.

state 24 hours after the anticoagulant has been given, there not only results a reduction in the intensity of the hypoprothrombinemia but the duration is also shortened (Fig 2). It is to be noted that the effect of the methylxanthine does not become detectable until 24 hours after its administration. If the methylxanthine is given 48 hours after the anticoagulant, the capacity to arrest the developing hypoprothrombinemia is nullified and there appears to be no effect on its duration. The curve in Fig 2 (labeled "Anticoagulant + theobromine 24 hrs apart") was constructed from sixteen separate trials with eight different dogs, in which the anticoagulant was first given at the level of 10 mg per kilo and theobromine at the 100 mg per kilo level.

TABLE V

*Resistance to 3,3'-Methylenebis(4-hydroxycoumarin) Caused by Single Dose of Theobromine in a Dog*

Date	Treatment	Prothrombin time (12.5 per cent)	
		48 hrs after administration	96 hrs after administration
1943		sec	sec
Jan 10	10 mg anticoagulant per kilo	89	106
" 24	10 " " " + 100 mg theobromine per kilo	31	35
Feb 7	10 mg anticoagulant per kilo	45	35
" 14	10 " " " "	48	45
Mar 7	10 " " " "	33	26
" 21	15 " " " "	37	27
May 23	15 " " " "	66	96
July 6	15 " " " "	77	96

\* The average normal 12.5 per cent plasma prothrombin time of this dog was 25 ( $\pm 2$ ) seconds at each date prior to the test.

*Effect of Daily Ingestion of Methylxanthines on Survival Time of Rats Receiving 3,3'-Methylenebis(4-hydroxycoumarin)*<sup>6</sup>—The conditions for the survival tests are essentially those given in previous papers in this series (12, 24). The results of many trials are herein summarized. The control rats maintained on the semisynthetic diet (12) survived a daily intake

\* The effect of the substances listed below on the action of the anticoagulant in the rat was also tested. They were either given with the anticoagulant or 12 hours later at the 50 or 100 mg level. Adenine, uric acid, allantoin, xanthine, arginine, xanthopterin, guanidine, methylguanidine, dimethylguanidine, urea, creatine, creatinine, glycoeyamine, uracil, and 6-methyluracil. Some of these compounds afforded a detectable protection against the induced hypoprothrombinemia, but their activity does not approach that of theobromine, caffeine, and theophylline.



of 2 mg of the anticoagulant for about 13 to 15 days. When 100 mg of theobromine were added to the ration in addition to the anticoagulant, the average survival time was more than 60 days.<sup>7</sup> With a daily intake of 50 mg of caffeine the survival time was also over 60 days. In sharp contrast, theophylline at the 50 mg level offered only slight protection, the survival time being 17 days. Likewise xanthine at the 100 mg level showed only a slight protection. The effect of uric acid, arginine, allantoin, urea, and guanidine was also tested. These compounds are devoid of the capacity to influence survival when the anticoagulant is ingested daily.

#### DISCUSSION

Control trials indicated that the methylxanthines do not affect the prothrombin time when added to blood or plasma *in vitro*. The hyperprothrombinemic effect is not due to hemoconcentration resulting from diuresis, or to replacement of the antihemorrhagic naphthoquinones in the synthesis of prothrombin. It would therefore appear that the capacity to elaborate prothrombin must be affected directly. The most plausible rationalization that can be advanced is that a functional stimulation of hepatic tissue is brought about, just as in a contrary manner the action of 3,3'-methylenebis(4-hydroxycoumarin) most likely involves a functional inhibition of prothrombin synthesis.

This point of view has arisen from the study of certain other factors that affect the coagulation mechanism *in vivo*. Trials with the dog (unpublished) indicate that the methylxanthines reduce the hepatotoxic action of chloroform as reflected by the hypoprothrombinemia measured with 12.5 per cent plasma. Furthermore, we have found that the action of the methylxanthines in normal dogs is not restricted to increased prothrombin activity. A readily detectable increase in the plasma fibrinogen level also results. A full report of this work will follow.

The specific hyperprothrombinemic action of the multimethylated xanthines (caffeine, theobromine, and theophylline), as opposed to the inactivity of related purine derivatives (xanthine, uric acid, adenine, allantoin, glycoxyamine, urea, creatinine, methylguanidine, and dimethylguanidine), merits comment. The possibility that the activity of caffeine, theobromine, and theophylline might result from a release of methyl groups was explored (25). The administration of massive doses of choline and methionine to dogs (200 to 600 mg per kilo) alone, or together with large doses of xanthine, was tried. A significant effect on the prothrombin time was not observed. Similar trials with the rat were also essentially negative. In sum, the intact multimethylated xanthine molecule must be available for effective hyperprothrombinemic action. The border line

<sup>7</sup> The rats were in good condition and showed no hemorrhagic lesions when the test was terminated.

response realized with heteroxanthine (7-methylxanthine) tends to sustain this premise

One of the significant points that has been established since the anticoagulant 3,3'-methylenebis(4-hydroxycoumarin) has become available is that a primary relationship exists between thrombus formation and the clotting mechanism of the blood. The work of Dale and Jaques (26), Richards and Cortell (27), Bollman and Preston (28), and Meyer and coworkers (29) on experimental thrombosis done with 3,3'-methylenebis(4-hydroxycoumarin) established that effective reduction of extravascular and intravascular thrombus formation parallels the diminished plasma prothrombin level (or activity) with its associated hypocoagulability.

The methylxanthines are extensively used in the treatment of cardiovascular disorders in man in which the threatening complication is frequently thrombus formation (14, 30). The dose of methylxanthine used is of a relatively high order and it is recommended that they be given thrice daily and frequently for as long as 1 to 2 weeks continuously (Osler-Christian (14)). Since our results on such diverse species as the dog, rabbit, and rat indicate that the methylxanthines not only render the blood hypercoagulable, but also counteract such a potent hypoprothrombinemic agent as 3,3'-methylenebis(4-hydroxycoumarin), it is conceivable that their use in man might augment the tendency for thrombus formation.<sup>8</sup>

We feel that the reported findings merit consideration, especially since it was recently stated (32) in a frank evaluation of drugs used in the treatment of cardiovascular disorders, "It is evident then that there is considerable doubt as to whether or not aminophylline is of value as a coronary vasodilator in man. However, no investigator has found the drug to be dangerous when given by mouth, so it can, therefore, be safely assumed that even though the aminophylline may do the patient no good, it can certainly do him no harm." In the light of the findings presented the justification for this point of view may be questioned (33).

We wish to thank Dr. Carl Nielsen and Mrs. F. P. Dann, Abbott Laboratories, North Chicago, for independently assaying the methylxanthines for antihemorrhagic activity in the chick and Dr. R. S. Overman for collaborating with us in the early stages of this study.

#### SUMMARY

1. It is shown that single oral doses of the methylxanthines, theophylline, theobromine, and caffeine, induce in the dog, rabbit, and rat a state of

<sup>8</sup> The eminent pathologist Aschoff has written, "The view that increased coagulability of blood is an essential point for the production of thrombosis, has been strongly upheld, especially by clinical observers. The existence of this increased coagulability and the likelihood that it is a promoting factor, or, better, an accompanying phenomenon of thrombosis cannot be denied." (31) pp. 253-254.

hyperprothrombinemia This effect is not exhibited by other purines, pyrimidines, and related compounds Choline and methionine are likewise devoid of this action

2 The hyperprothrombinemia resulting from a single dose of methylxanthine persists for 4 to 5 days in the dog Through repeated small doses dogs were maintained in the hyperprothrombinemic state for periods up to 30 days

3 The methylxanthines can counteract the hypoprothrombinemic action of the anticoagulant 3,3'-methylenebis(4-hydroxycoumarin) in the dog When they are given either with, or 24 hours after, the anticoagulant, they not only reduce the intensity of the hypoprothrombinemic response but also shorten its duration

4 Single doses of the methylxanthines will protect a standardized dog against repeated doses of the anticoagulant for periods up to 14 weeks Continued ingestion of caffeine and theobromine prolonged the survival time of rats fed the anticoagulant daily

5 It is suggested that the methylxanthines produce a functional stimulation of hepatic tissue, which accounts for the hyperprothrombinemia in normal animals and for the protective action against the anticoagulant

6 A possible bearing of these findings on the use of methylxanthines in conjunction with cardiovascular therapy is suggested

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# NOTE ON HYPERPROTHROMBINEMIA INDUCED BY VITAMIN K\*

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Dam and Glavind (1) reported that feeding chicks large quantities of a vitamin K concentrate prepared from spinach did not make the coagulability of the blood plasma "supernormal." Adams (2) came to the same conclusion with trials on the rabbit and man. Allen reported (3) that the injection of 200 mg of the water-soluble tetrasodium-2-methyl-1,4-naphthohydroquinone diphosphoric acid ester (synkayvite, Roche) into two human subjects did not produce abnormally shortened prothrombin times. In a recent review on prothrombin response to vitamin K therapy, Allen wrote ((4) p 403) "it does not seem to be possible to elevate the prothrombin to levels above normal with doses even far in excess of the current amount used."

In contrast to this point of view, several clinical investigators have indicated that in the treatment of hemorrhagic conditions correctable with vitamin K, the restored prothrombin levels temporarily exceeded the preexisting normal values subsequent to the administration of vitamin K ((5) Fig 11, p 65), ((6) Fig 2, p 220). This is in agreement with our observations on the protective action of 2-methyl-1,4-naphthoquinone on the hypoprothrombinemia induced by the anticoagulant 3,3'-methylenebis(4-hydroxycoumarin) in the rat (7). It was found that large doses of the naphthoquinone not only protected the rat completely against the action of the anticoagulant, but induced a temporary state of hyperprothrombinemia. Recently Mikhlin (8) has also indicated that vitamin K<sub>1</sub> and K<sub>2</sub>, as well as all related synthetic products, are without effect on the blood of normal animals and man. However, Mikhlin (8, 9) and Babuk (10) report that a vitamin K concentrate prepared from maize stigmas accelerated the normal coagulability of the blood in the dog, rabbit, rat, and man. They attributed the hypercoagulable effect to elevated plasma prothrombin levels.

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The purpose of this report is to indicate that the administration of 2-methyl-1,4-naphthoquinone in large single oral doses to the dog, rabbit, and rat increases the prothrombin level (or activity) above the pre test normal values. The resulting hyperprothrombinemia persists for several days, depending on the dose. Comparable observations have been made independently on the dog by Dr R K Richards, Abbott Laboratories, North Chicago, Illinois, and on man by Dr S Shapiro, Welfare Hospital, New York University Division III, New York (private communications).

TABLE I

*Effect of 2 Methyl 1,4 naphthoquinone on the Prothrombin Time from Different Species\**

Species	Naphthoquinone fed mg per kg	Prothrombin time in sec of 12.5 per cent plasma							
		Normal and standard deviation	12 hrs after feeding	24 hrs after feeding	48 hrs after feeding	72 hrs after feeding	120 hrs after feeding	144 hrs after feeding	168 hrs after feeding
Rat	4	40.0 ± 1.0			40.5	40.3			
	20	40.0 ± 1.0		41.0	36.9	35.0			
	40	39.3 ± 1.0	39.9	40.7	36.3	36.0			
Dog	5	22.8 ± 0.3	22.5	21.5	23.0	23.1	23.2		
	10	20.0 ± 0.5	17.0	17.2	18.0	20.0			
	25	30.5 ± 0.9	26.8	24.0	24.2	25.6	29.2		
	50	32.3 ± 0.8	29.0	22.0	22.0	24.7	26.0	27.7	32.0
Rabbit	1	21.7 ± 0.7	22.0	20.2	20.7	20.3		21.2	
	5	25.0 ± 0.2	22.0	20.2	21.0	24.2	24.5		
	10	23.9 ± 0.5	20.2	21.0	22.7	22.3	22.7	24.5	
	25	25.7 ± 0.6	24.4	19.7	20.2	22.5	22.3	22.5	26.5

\* Each line of figures represents a series of values obtained with a single animal.

#### EXPERIMENTAL

The details of the animal experiments have already been described (7, 11-13). In this study, as in all the previous work, whole plasma (100 per cent) and the 50, 25, 12.5, and 6.25 per cent concentrations were routinely explored for prothrombin level (or activity). For reasons previously emphasized the data will be restricted to the plasma concentration of 12.5 per cent (1 part plasma, 7 parts saline solution). Plasma dilution curves comparable to that of Fig 1 (13) were obtained. The hyperprothrombinemia was observed with both 2-methyl-1,4-naphthoquinone and the water-soluble 2-methyl-1,4-naphthoquinone bisulfite (hykinone, Abbott).<sup>1</sup> The data presented are restricted to the results

<sup>1</sup> Hykinone, Abbott, was kindly supplied by Dr Carl Nielsen, Abbott Laboratories, North Chicago, Illinois.

obtained with the first compound. Representative results realized with the dog, rabbit, and rat are given in Table I. No toxic symptoms were noted during the course of these trials.<sup>2</sup> The minimum single oral dose at which a consistent hyperprothrombinemic effect could be detected under our conditions in the rabbit was approximately 5 mg per kilo, in the dog 10 mg per kilo, and in the rat 20 mg per kilo.<sup>3</sup>

## SUMMARY

The oral administration of 2-methyl-1,4-naphthoquinone to the dog, rabbit, and rat in large doses induces a state of hyperprothrombinemia readily detected with 12.5 per cent plasma, which persists for several days, depending on the dose.

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<sup>2</sup> The oral lethal dose of various naphthoquinones ranges from 200 to 500 mg per kilo (14, 15).

<sup>3</sup> In a discussion of this subject with Dr. J. Garrott Allen, Department of Surgery, Billings Hospital, University of Chicago, he informed us that the largest dose of Synkayvite that he used approximated 4 mg per kilo. It would appear from our results that the dosage used by Dr. Allen was just below the level at which the hyperprothrombinemic effect becomes detectable.





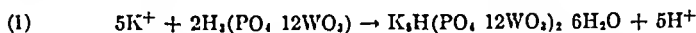
# MICRODETERMINATION OF POTASSIUM BY PRECIPITATION AND TITRATION OF THE PHOSPHO-12-TUNGSTATE

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In the present method potassium is precipitated as the difficultly soluble salt of phospho-12-tungstic acid (for which the abbreviation PTA will be used) The precipitation under the conditions employed can be expressed as



The formula given for the potassium phosphotungstate, with 6 molecules of  $H_2O$ , indicates the composition of the precipitate dried at  $100^\circ$

The precipitate can be measured either by weighing, or by titrating with alkali to an end-point at pH 9 The reactions in the titration are indicated by the equation,



On the right side B represents either K or Na The high alkali requirement of 9.4 equivalents of NaOH for 1 equivalent of K provides favorable conditions for microtitration

For potassium determinations on ash of biological material the phospho-12-tungstic acid has furthermore the advantage that it does not form insoluble compounds with Ca, Mg, Na, or Fe, nor is the analysis affected by any but gross amounts of  $PO_4$ , consequently the potassium can be precipitated without preliminary removal of any of the other constituents usually encountered in the ash of blood, urine, or tissues

The phospho-12-tungstic acid precipitates various organic nitrogenous substances, such as the basic amino acids, alkaloids, peptone, proteins, uric acid, and ammonia However, these substances are removed by ashing

That potassium forms a difficultly soluble salt of one of the phosphotungstic acids was observed by Gibbs (1) Pechard (2) stated that addition of potassium salts to a solution of sodium phospho-12-tungstate produced an amorphous precipitate of a potassium salt which was insoluble in cold water, slightly soluble in hot Nikitina (3) obtained a salt of which the anhydrous form was  $K_5 \cdot PO_4 \cdot 12WO_3$ , and stated that it was soluble in water and insoluble in strong HCl solution Nikitina stated that the phosphotungstate could be used to determine potassium in the absence of

sodium, or to detect potassium in the presence of sodium. Aside from these statements of Nikitina there appears to be little in the literature to indicate that the phosphotungstate might be useful in determination of potassium.

The reason for the previous non-use of PTA in quantitative potassium determination probably lies in peculiarities of the potassium phosphotungstate precipitate. Complete precipitation of the last traces of potassium from solution requires prolonged standing, and the precipitate is so fine that it is not retained by ordinary filters of paper, asbestos, or glass. We have found, however, that evaporating the solution of potassium salt and PTA to dryness makes the precipitation quick and complete. The difficulties of filtration have been avoided by washing by centrifuge, for which the heavy gravity of the precipitate makes it well adapted.

Unlike the chloroplatinate method for precipitating potassium, the phosphotungstate method does not require excess of precipitating reagent to prevent precipitation of sodium salts.

In the present paper the titrimetric procedure based on Equation 2 is described. In the accompanying paper (4) a gravimetric method is outlined, in which the potassium phosphotungstate precipitate is weighed on a semimicro balance.

### Apparatus

*Platinum crucibles* Platinum crucibles, preferably of 8 to 12 cc capacity (15 cc permissible), are desirable for ashing. The inside of the crucible should be smooth and clean. Platinum etched by ignition of organic phosphates has been found to give low results.

*Silica crucibles* (8 to 12 cc) or *silica centrifuge tubes* (15 cc) may be used as less desirable substitutes for platinum crucibles.

*Centrifuge tubes* 15 cc conical Pyrex centrifuge tubes are used. They should be of such internal diameter at the bottom that 0.2 cc of water will make a column 5.5 to 7.5, preferably 6 to 7, mm high, measured from the bottom of the meniscus to the middle of the arc which forms the floor of the tube. If the tube is too narrow at the bottom, it will be difficult to boil alkaline solution in it without spurling. On the other hand, if the tube is too wide at the bottom the precipitate does not pack as well for washing. Graduated tubes are used, or ungraduated tubes marked with hydrofluoric acid or diamond pencil at 0.2 and 0.5 cc levels. Before use the tubes are cleaned with chromic acid, and then washed thoroughly with water.

*Burettes* Two small burettes are used, one for sodium hydroxide and one for sulfuric acid. The 2 or 3 cc Bang ((5), (6) p 13) micro burettes with reservoirs are most convenient,<sup>1</sup> but 5 cc burettes with 0.02 cc divi-

<sup>1</sup> These burettes, improved by Koch, were obtained from the Ace Glass, Inc., Vineland, New Jersey.

sions can be used. The reservoir of the Bang alkali burette is protected from atmospheric  $\text{CO}_2$  by a soda lime tube. It is desirable to use burettes delivering small drops. The size of the drops can be halved by greasing the sides of the burette tips with a thin film of vaseline mixed with a little caprylic alcohol.

*Suction capillary.* For removal of wash water from the centrifuged precipitate a Pyrex glass tube of 4 to 6 mm diameter is drawn out to a strong capillary of about 12 cm length. The tip of the capillary is bent to a right angle over a micro burner, and the part beyond the bend is cut

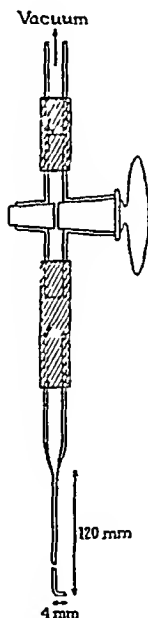


FIG. 1. Arrangement for washing potassium phosphotungstate precipitate

to a length of 3 to 4 mm. The capillary is attached to a glass cock as shown in Fig. 1, for accurate control of suction, and is connected with a suction flask.

*Arrangement for passing  $\text{CO}_2$ -free air through tubes during titration of potassium phosphotungstate.* Any arrangement providing a slow stream of  $\text{CO}_2$ -free air may be used. The arrangement used in our work was that of Van Slyke, MacFadyen, and Hamilton (7), in which a pair of connected 2 liter aspirator bottles partly filled with 10 per cent  $\text{NaOH}$  serves as reservoir for  $\text{CO}_2$ -free air under convenient pressure.

*Alundum pieces*<sup>2</sup> of 1 to 2 mm length to promote smooth boiling The pieces are washed with cleaning mixture and hot HCl, rinsed with water, dried, and kept in a small Petri dish, protected from dust

*Electric muffle* in which the temperature can be set at 460–480° for ashing organic matter

*Pyrex glass rods* of 2 to 3 mm diameter, some of 7 cm and some of 12 cm length The shorter rods are provided with tight rubber tips and are used for the transfer of the precipitate from the platinum crucibles to the centrifuge tubes The longer ones are used to stir up the precipitate

### Reagents

*Phospho-12-tungstic acid*<sup>3</sup> From mixture with other phosphotungstic acids the 12-acid is separated with ether by Winterstein's (8) method, which has been shown by Van Slyke, Hiller, and Dillon (9) to yield the 12 acid,  $H_3(PO_4 \cdot 12WO_3) \cdot 7H_2O$  The phosphotungstic acid mixture with the 12-acid predominating can be prepared as described by Wu (10) from tungstic and phosphoric acids, but we have found it convenient to use commercial preparations purified as follows

Commercial phosphotungstic acid was dissolved in an equal weight of water in a separatory funnel A volume of ether approximately equal to the volume of the solution was added and shaken The phospho-12-tungstic acid and ether formed a heavy oil which settled to the bottom It was washed three times with water, a volume about equal to that of the oil being used for each washing The washed ether solution was then evaporated, and the residue dried, at room temperature in a vacuum desiccator protected from light (Evaporation on a steam bath yields a product which leaves a detectable residue in blank analyses by the present method, this residue would cause high titration blanks) The dried PTA was stored in brown bottles The yield of the PTA from different commercial products varied from 30 to 80 per cent

The purified PTA should be white, and 1 gm should dissolve without turbidity in 5 cc of distilled water without heating

*4 per cent solution of PTA* A water solution containing 4 gm of purified PTA per 100 cc is kept in a brown glass-stoppered bottle, preferably in an ice box The solution keeps thus for months without becoming visibly turbid It is, however, advisable as a precaution to centrifuge before use the amount needed for a series of analyses

*0.8 per cent solution of PTA* By 5-fold dilution of the 4 per cent stock solution

<sup>2</sup> The alundum pieces are size No. 14 of the Norton Company

<sup>3</sup> An excellent preparation of the phospho-12-tungstic acid for use in the present method is prepared by the Anachemia Company, 70 East 45th Street, New York This product meets all the requirements, and needs no further purification

*Thymol blue-phenolphthalein mixed indicator solution* 50 mg of thymol blue are dissolved in 21.5 cc of 0.01 N NaOH and diluted to 50 cc with water. 50 mg of phenolphthalein are dissolved in 50 cc of absolute alcohol. Equal volumes of the two solutions are mixed.

*Approximately 4 N sulfuric acid* 11 cc of concentrated sulfuric acid diluted to 100 cc

*15 per cent solution of anhydrous sodium sulfate*

*Approximately 0.1 N hydrochloric acid*

*0.04 or 0.02 N standard sulfuric acid*

*1.0, 0.04, and 0.02 N NaOH solutions, approximately CO<sub>2</sub>-free* The 1 N NaOH is prepared from a saturated sedimented NaOH solution as described by Peters and Van Slyke ((6) p. 29), is standardized, and is kept in a paraffin-lined bottle protected from atmospheric CO<sub>2</sub>. The 0.04 and 0.02 N solutions are prepared by dilution from the stock 1.0 N solution, and are standardized by titration against the 0.04 or 0.02 N sulfuric acid, with use of the same indicator and end-point employed in the titration of potassium phosphotungstate. In preparing the 0.02 and 0.04 N from the 1.0 N solution the volumetric flasks in which the dilutions are made are nearly filled with water, before the 1.0 N NaOH is added, in order to minimize absorption of atmospheric CO<sub>2</sub> by the solutions in the flasks.

#### I ANALYSIS OF 1 CC SAMPLES OF SERUM OR PLASMA

*Ashing of Sample*—1 cc of serum or plasma<sup>4</sup> is pipetted into a small platinum crucible of 8 to 12 cc capacity. From a Mohr pipette 0.5 cc of approximately 4 N H<sub>2</sub>SO<sub>4</sub> is added and the contents are mixed by gentle agitation. The crucible is placed uncovered for 1 to 2 hours on a boiling water bath, or is covered with a Pyrex watch-glass<sup>5</sup> and placed in an oven at a temperature not exceeding 100°. After as much water as possible has been driven off at 100°, the crucible, still covered with a Pyrex watch-glass, is heated on a hot-plate until the charred residue is dry.

<sup>4</sup> In order to avoid diffusion of potassium from cells to either serum or plasma the latter should be separated at the earliest possible moment after blood is drawn. The speed of diffusion of potassium from red cells has been studied in detail by Gerschman (11). Other precautions to be taken have been outlined by Peters and Van Slyke ((6) p. 62) and by Weichselbaum, Somogyi, and Rusk (12). Heparin used as anticoagulant in excessive amounts has been reported to favor diffusion of potassium from the red blood cells (13).

<sup>5</sup> The watch-glass prevents loss from spattering of the concentrating mixture. If spattering occurs, the particles are visible on the glass, and are rinsed back with water into the crucible.

Instead of evaporation on a steam bath, the water can advantageously be driven off by heat from above, from an "Infra Radiator," manufactured by the Fisher Scientific Company of Pittsburgh. The crucible is left uncovered when thus treated, and the evaporation is finished in about an hour, without spattering.

The crucible is then uncovered and placed in a cold muffle, where the temperature is raised to between  $460^{\circ}$  and  $480^{\circ}$ . Below  $460^{\circ}$ , ashing might not be complete, above  $480^{\circ}$  some volatilization of potassium may occur. The crucible is left in the furnace at  $460$ – $480^{\circ}$  for a period sufficient to produce a uniform white ash. An overnight ignition was found satisfactory.

If a muffle furnace is not available, ashing may be carried out over a micro burner as described by Shohl and Bennett (14).

*Dissolving the Ash and Precipitating the Potassium*—After the material is ashed, the walls of the crucible are washed down with 0.5 cc of 0.1 N HCl, and the ash is dissolved in this solution. Then 0.5 cc of the 4 per cent solution of purified PTA is added dropwise. The contents are mixed and the crucible is placed on a steam bath. It is removed as soon as the contents are dry. If the crucible is left on the bath 10 or 20 minutes longer, results are not affected. But, if left for 2 hours, traces of the free PTA may become insoluble, and cause high titration results.

*Transfer and Washing of Potassium Phosphotungstate Precipitate*—Three successive washings suffice to remove all the excess PTA. The water used for the first washing serves also to transfer the precipitate from the crucible to a centrifuge tube.

To the dry precipitate in the cooled crucible 2 cc of water are added. A thin film of vaseline is applied to the edge of the crucible. Precipitate and water are stirred up with a small rubber-tipped rod, and the suspension is poured into a 15 cc centrifuge tube. Four more 2 cc portions of water are similarly stirred up with the precipitate and transferred to the centrifuge tube. An additional 2 cc of water are used to rinse the rubber tip of the rod, bringing the total volume of water in the tube to 12 cc. The tube is centrifuged at 3000 R P M for at least 15 minutes.<sup>7</sup>

The supernatant fluid is then removed by suction with a capillary (Fig 1). Before suction is turned on, the tip of the capillary is immersed 1 to 2 cm below the surface of the supernatant fluid. Suction is regulated by gentle turning of the stop-cock. During removal of the wash water, care is taken that the tip stays well submerged in order to avoid removal of any

<sup>7</sup> For complete precipitation it is desirable to use at least 20 to 30 per cent excess of PTA, viz., 40 to 50 mg of PTA per 1 mg of potassium. Greater excess increases the blank slightly, but does not otherwise affect results. The 20 mg of PTA added suffice to precipitate 0.6 mg of potassium, which is 3 times the normal 0.2 mg of K in 1 cc of serum. For analyses of the rare pathological sera with more than 0.6 mg of K per cc, 40 mg of PTA are used.

When many analyses are carried out, it is advantageous to run them in sets of eight. While one set is in the centrifuge, the precipitates of another set can be transferred to tubes, or the wash water over previously centrifuged precipitates can be changed.

precipitate that may float on the surface. The fluid is removed until its meniscus reaches the 0.5 cc mark near the bottom of the tube. The stop-cock is then turned off and the capillary lifted above the surface. The tip of the capillary is washed with a few drops of distilled water, which are drained down into the tube.

For a second washing 5 cc of water are run down the walls of the tube and thoroughly stirred up with the precipitate. To insure complete washing a homogenous suspension should form in this operation. The rod is withdrawn and washed with 2 cc of water, bringing the total volume used in this washing to 7 cc. The contents of the tube are centrifuged for 10 minutes. The supernatant fluid is removed, to the 0.2 cc mark this time.

A third washing is carried out in the same way as the second, with 7 cc of water.

Rigorous adherence to the washing technique is necessary for accurate results. The volumes of water used should be measured, preferably from a burette, not only to assure suitable amounts for complete removal of the excess PTA, but also to assure that the amount of precipitate dissolved in the washing is the amount assumed in the solubility correction of the calculation formulae (see "Calculation"). The total volume of water used for transfer and washing should be 26 cc.

#### *Titration of Potassium Phosphotungstate*

The titration is carried out as by Toennies and Elliott (15) in analyses of PTA. One first adds an excess of standard NaOH and heats to change all the phosphotungstate to  $\text{Na}_3\text{PO}_4$  and  $\text{Na}_2\text{WO}_4$ . Then excess acid is added and the solution is boiled to remove traces of  $\text{CO}_2$  absorbed from the air during the alkali treatment. Finally alkali is added again until the end-point is reached at pH 9 (see Equation 2).

First a piece of alundum is added to the washed precipitate in order to promote smooth boiling. Then from the alkali burette about 1 cc of 0.04 N NaOH is added. The contents of the tube are mixed by tapping the side of the tube, and are heated over a micro burner until boiling begins. The burner is then removed and more NaOH is added to the hot solution until the precipitate is nearly dissolved. 1 drop of the mixed thymol blue-phenolphthalein indicator is now added, and is followed by more alkali until a blue-violet color becomes permanent. Thereupon 0.3 to 0.5 cc more of the alkali is added and the solution is boiled again, for at least 30 seconds. If the color of the solution changes from the blue-violet, more alkali is added and the 30 second boiling is repeated.

From the acid burette enough 0.04 N sulfuric acid is now added to turn the indicator yellow, then a few more drops of acid are added, and the solution is brought to boiling for half a minute to expel  $\text{CO}_2$ .



Finally, without waiting for the solution to cool, 0.04 N NaOH is added to change the color through blue-green back to the violet of the end point. During this last addition of the alkali, in order to minimize absorption of atmospheric CO<sub>2</sub>, the mouth of the centrifuge tube is closed with a perforated rubber cap and the tip of the burette is inserted through the perforation. Or, better, a stream of CO<sub>2</sub>-free air is run through the tube, as described below for analysis of 0.2 cc portions of serum.

The titration should be done with the tube against a white background, and preferably in daylight or in artificial light rich in blue. The color of the indicator changes from acid-yellow through blue-green to a final violet at pH 9. NaOH should be added until a distinct violet appears and stays for at least 1 minute. The end-point is sharp to about 0.005 cc of the 0.04 N alkali.

As a standard for controlling the end-point color, one may prepare a tube containing 0.1 per cent Na<sub>2</sub>HPO<sub>4</sub>, the same volume as the titrated solution, with the same amount of indicator, and at the same temperature. The end-point at pH 9 is not the deepest color of the indicator, and one may overrun it unless a control standard is used, until one is familiar with the color change. After that the control tube is usually unnecessary.

If the end-point should be overrun by addition of too much alkali, enough 0.04 N sulfuric acid is added to turn the solution yellow, the solution is boiled again for 30 seconds, and the titration to the violet end-point is repeated.

The reading on the alkali burette, minus the reading on the acid burette, indicates the volume of 0.04 N NaOH required to titrate the precipitate.

**Blank Determination**—Blank analyses are run in which water replaces the serum. If the PTA is properly prepared, so that solutions of it leave no insoluble residue when evaporated to dryness as in the analysis, the blank is constant and small. The values obtained in forty-three blank determinations with various PTA solutions averaged 0.024 cc of 0.04 N NaOH. For a normal serum containing 5 milliequivalents of potassium per liter this blank would amount to 2 per cent of the NaOH used in the titration. With long standing of the 4 per cent PTA reagent solution the blank may increase. Hence blank analyses should be repeated occasionally, and whenever a new 4 per cent PTA solution is prepared.

**Calculation**—The potassium obtained in the sample analyzed is calculated as follows

$$Mg\ K\ in\ sample = 0.1657 (T - B) + 0.0090$$

$$" \quad " \quad per\ 100\ cc\ plasma = 16.57 (T - B) + 0.90$$

$$Milliequivalents\ K\ per\ liter\ plasma = 4.24 (T - B) + 0.23$$

$T$  = the cc of 0.04 N NaOH to titrate the potassium phosphotungstate  
 = the cc of 0.04 N NaOH added from the alkali burette minus the cc of

0.04 N  $\text{H}_2\text{SO}_4$  added from the acid burette  $B =$  the cc of 0.04 N NaOH to titrate the blank similarly. The addition of 9  $\gamma$  to the K titrated in the sample or 0.9 mg per 100 cc of serum, or 0.23 milliequivalents per liter of serum, in the last terms of the respective formulae, is a correction for the solubility of the potassium phosphotungstate in the 26 cc of wash water.

The factor 0.1657 indicates 9.44 equivalents of NaOH per equivalent of K. This experimentally based factor differs slightly from the theoretical factor 9.40 indicated by Equation 2.

## II ANALYSIS OF 0.2 CC SAMPLES OF SERUM OR PLASMA

*Ashing Sample and Dissolving the Ash*—0.2 cc of serum or plasma is pipetted into an 8 to 12 cc platinum crucible. 3 drops of approximately 4 N sulfuric acid are added. The drying and ashing are as described for analysis of 1 cc of serum. The walls of the crucible are then washed down with 0.2 cc of 0.05 N HCl, in which the ash is dissolved.

*Precipitation of Potassium Phosphotungstate*—0.5 cc of 0.8 per cent PTA solution is run down the walls of the crucible to the ash solution, and the mixture is evaporated to dryness on a steam bath or in a vacuum desiccator, as described for 1 cc samples.

*Transfer and Washing of Precipitate*—To the dried precipitate in the platinum crucible, 1 cc of water is added. Vaseline is applied to the edge of the crucible and the contents are stirred up with a small rubber-tipped glass rod and transferred quantitatively to a 15 cc centrifuge tube with 5 cc of water added in 1 cc portions. The rubber tip is washed with an additional 1 cc of water, bringing the total of water used for the transfer to 6 cc. The tube is centrifuged for 10 minutes at 3000 r.p.m. The supernatant fluid is then removed in the manner described for analysis of 1 cc of plasma, except that the fluid is removed to the 0.2 cc mark. For the second washing of the precipitate 1 cc of water is added to the tube. The contents are stirred up with a slender glass rod, and 1.0 cc of water is used to rinse the rod. The suspension is centrifuged for 10 minutes at high speed. The supernatant fluid is again removed to the 0.2 cc mark. A third washing is carried through in the same manner with another 2.0 cc of water. The total volume of water used in washing and transfer should be 10 cc.

*Microtitration of Potassium Phosphotungstate*—A single small piece of alundum is added to the tube. Enough 0.02 N NaOH is added to produce nearly complete solution of the precipitate with boiling, as described for the analysis of 1 cc of plasma. When the solution has turned nearly clear, 1 drop of mixed indicator solution is added, then enough 0.02 N NaOH to produce a strongly alkaline reaction, then 0.3 to 0.5 cc additional excess of the NaOH. The alkaline solution is boiled for 30 seconds to complete

the splitting of the phosphotungstate From the acid burette 0.02 N sulfuric acid is added until the indicator turns acid, then a few drops of excess acid, and the  $\text{CO}_2$  is expelled by boiling for 1 minute

Without permitting the solution to cool, a stream of  $\text{CO}_2$ -free air is started through the tube, and the excess acid is titrated with 0.02 N NaOH, the tip of the alkali burette being kept close to the surface of the solution, the end-point is as described for analysis of 1 cc of serum

*Blank Determination*—With each series of analyses a blank determination is run, 0.2 cc of water being used instead of serum

*Calculation of Results of Analysis of 0.2 Cc of Plasma*—

$$\text{Micrograms K in sample} = 82.9 (T - B) + 3.5$$

$$\text{Mg K per 100 cc plasma} = 41.45 (T - B) + 1.75$$

$$\text{Milliequivalents K per liter plasma} = 10.61 (T - B) + 0.45$$

The last term of each equation is a solubility correction  $T$  and  $B$  indicate cc of 0.02 N NaOH used to titrate the precipitate and the blank respectively

### III ANALYSIS OF WHOLE BLOOD

To 1 cc of whole blood in a platinum crucible add 0.5 cc of 4 N sulfuric acid and 5 drops of a 15 per cent solution of  $\text{Na}_2\text{SO}_4$ . The mixture is then concentrated and ashed as described for serum. The ash is taken up with 0.3 cc of concentrated HCl and the solution is evaporated to dryness. The residue is dissolved in 0.1 N HCl, and the solution is washed into a 10 cc volumetric flask, 0.1 N HCl being used for the washing. The solution is made up to volume, and aliquots of 2 cc, equivalent to 0.2 cc of blood, are used for precipitation with PTA. Precipitation, washing, and titration are as described for 1 cc portions of serum

*Calculation*—

$$\text{Mg K in sample} = 0.1657 (T - B) + 0.0090$$

$$\text{" " per 100 cc blood} = 82.85 (T - B) + 4.5$$

$$\text{Milliequivalents K per liter blood} = 21.2 (T - B) + 1.15$$

### IV ANALYSIS OF URINE

A sample of urine containing 0.3 to 0.8 mg of potassium is pipetted into a platinum crucible. The desired amount of potassium is usually contained in 0.0001 of a 24 hour human (adult) urine specimen, or 0.1 to 0.5 cc of urine of ordinary concentration. 1 drop of a 15 per cent solution of potassium-free  $\text{Na}_2\text{SO}_4$  is added, and 0.3 cc of approximately 4 N sulfuric acid. From this point the procedure outlined for analysis of 1 cc samples of plasma is followed, except for four details

1 Before the final re-solution of the ash in 0.5 cc of 0.1 N HCl, the ash

is covered with 3 drops of concentrated HCl and evaporated to dryness. This treatment has been found essential for quantitative results when much phosphoric acid is present.

2 After re-solution of the residue in 0.5 cc of 0.1 N HCl, 1 cc, instead of 0.5 cc, of 4 per cent PTA is added, in order to cover the wider range of potassium encountered in urine analyses.

3 To titrate the larger amounts of potassium that may be found, a 5 cc burette is preferable to the 2 cc burette recommended for serum analyses.

4 The amount of PTA used will precipitate a maximum of 1.2 mg of K, requiring 7.23 cc of 0.04 N NaOH. If more than 6 cc are used in the titration, the analysis is repeated with a smaller sample.

*Calculation of Urine Potassium—*

$$\text{Mg K in sample} = 0.1657 (T - B) + 0.0090$$

$$\text{Gm " per liter urine} = \frac{0.1657 (T - B) + 0.0090}{\text{cc urine in sample}}$$

$$\text{Milliequivalents K per liter urine} = \frac{4.24 (T - B) + 0.23}{\text{cc urine in sample}}$$

*T* indicates cc of 0.04 N NaOH required to titrate the precipitate, *B* the cc required to titrate the blank.

#### V FECES

Of the dried and pulverized feces a sample of 1 to 2 gm is weighed into a silica dish free from etching, and is ashed in a muffle at 460° (It is inadvisable to ash the feces at this stage in platinum, because there may be so much phosphorus mixed with the organic material that the platinum would be etched). The ash is taken up in the smallest amount of concentrated HCl which will effect ready solution. The solution in the dish is evaporated to complete dryness in an oven at 110° to hydrolyze metaphosphates.

The ash is dissolved in hot 0.1 N HCl and rinsed with about 50 cc of 0.1 N HCl into a 100 cc volumetric flask. The solution is cooled and 2 or 3 drops of 1 per cent phenolphthalein are added. To precipitate phosphate just enough pulverized calcium hydroxide<sup>8</sup> is added to turn the phenolphthalein deep red. The solution is then shaken repeatedly, and is let stand at least 30 minutes. Then an excess of saturated ammonium oxalate solution, 10 cc or more, is added to precipitate the dissolved calcium, the mixture is shaken, diluted to 100 cc, mixed, let stand a short time, and then is centrifuged or is filtered through a dry No. 42 Whatman filter paper.

<sup>8</sup> The Ca(OH)<sub>2</sub> should be reagent grade, and must be tested for potassium by using it in blank analyses. If small amounts of potassium are present, they may be removed by suspending the Ca(OH)<sub>2</sub> in water and then washing repeatedly on a filter

Of the filtrate, samples containing between 0.2 and 1.0 mg of potassium are pipetted into platinum crucibles. The solution is treated with  $\text{Na}_2\text{SO}_4$ , ashed, and otherwise handled as described above for analysis of urine.

## VI. TISSUES

Weighed samples of tissue containing between 0.15 and 1.10 mg of potassium are dried in platinum crucibles in an electric oven at 90–95°. After addition of 1 drop of a 15 per cent solution of potassium-free sodium sulfate the whole ashing, precipitation, washing, and titration procedure is carried out as in the case of urine samples.

With this micro procedure the small samples of muscle tissue obtained by the biopsy technique of Shank and Hoagland (16) can be satisfactorily analyzed. Removal of phosphate from the ash solution is not necessary, if the ash is evaporated to dryness with a few drops of concentrated  $\text{HCl}$  at 110°.

When the fat content of the tissue is considerable, the addition of  $\text{Na}_2\text{SO}_4$  is omitted and the procedure of Ferrebee, Parker, Carnes, Gerity, Atchley, and Loeb (17) followed for removal of fat and phosphate. Larger samples are needed in this case. Simultaneous sodium determinations can be carried out.

### *Derivation of Calculation Formulae*

The constants used in the formulae are derived from numerous analyses of standard potassium solutions. From solutions of purified  $\text{KCl}$  or  $\text{K}_2\text{SO}_4$  amounts containing from 0.1 to 1.0 mg of  $\text{K}$ , for the conditions used in analyses of 1 cc plasma samples, and from 20 to 120  $\gamma$  for the conditions used in analyses of 0.2 cc samples were pipetted into platinum crucibles and evaporated to dryness. For each amount triplicate or quadruplicate determinations were carried out by the procedures described above. The titration figures thus obtained were plotted against the known amounts of potassium. Linear curves of the type  $y = ax + b$  were obtained, where  $y$  = micrograms of  $\text{K}$  and  $x$  = cc of 0.04  $N$   $\text{NaOH}$  solution. The gradient,  $a$ , was found to be 165.7.

For the curve obtained under the conditions outlined for analysis of 1 cc of serum, the intersection with the ordinate indicated a  $b$  value of  $9 \pm 0.5 \gamma$  of potassium. This represents the amount of potassium dissolved as the phosphotungstate during the washing of the precipitate. It approximates the amount that the 26 cc of wash water can dissolve, as estimated from independent determinations of the solubility of potassium phosphotungstate (4).

The constants for use with serum samples of 0.2 cc were similarly obtained.

## VII ANALYSES WITH ASHING IN SILICA CRUCIBLES OR TUBES

If platinum crucibles are not available for ashing samples, clear, unetched silica crucibles or tubes may be used as less desirable substitutes. When serum was ashed at  $180^{\circ}$  in silica instead of platinum a mean loss of 4.6 per cent of the potassium occurred, apparently as the result of fusion of that proportion of the serum potassium with the silica (see Tables II and III). The loss was sufficiently variable to increase the standard deviation from the mean in series of analyses of identical serum samples to 2.9 per cent, compared with 1.2 per cent observed when the ashing was in platinum. When the temperature of ashing in silica was raised to  $515^{\circ}$ , the loss and variability increased. Consolazio and Talbott (18) have ashed in silica tubes at " $450-500^{\circ}$ " without apparent loss. It is possible that if a temperature in the neighborhood of  $450^{\circ}$  instead of  $480^{\circ}$  were used the loss might be less than 4 per cent. With ashing under the conditions prescribed in this paper, however, an empirical correction must be made in the calculation, and in about one analysis out of twenty an error exceeding  $\pm 5$  per cent may be statistically expected.

With silica crucibles the technique is exactly the same as when the ashing is done in platinum crucibles.

With a silica centrifuge tube the entire analysis, from ashing to titration, is run in the tube, without the transfer from crucible to tube that is necessary when a crucible is used for the ashing. Each tube should hold at least 14 cc, and should have a conical bottom of such diameter that 0.2 cc of fluid makes a column 5.5 to 7.7 mm high, preferably 6 to 7 mm, as specified above for Pyrex tubes. The tubes are provided with marks at levels indicating 0.2 and 0.5 cc content.

Each tube is charged with a sample and some 4 N sulfuric acid, as in analyses in platinum, and the material is dried. Drying may be accomplished by heating 1 to 3 hours on a steam bath, then overnight in an oven at  $100^{\circ}$ . Or the entire drying may be accomplished more rapidly on a steam bath if the tube is tilted somewhat and a stream of dust-free air is drawn through the tube by means of a glass capillary, which does not extend into the tube far enough to touch the liquid.

Ashing, re solution of the ash, addition of PTA, and evaporation to dryness to complete the precipitation of potassium phosphotungstate are as when the ashing is done in a crucible. However, the concentration to dryness in the tube may be accelerated by a stream of air, as described in the preceding paragraph. The tube is removed from the steam bath as soon as evaporation is complete. The precipitate is washed and titrated in the tube, as previously described.

*Calculation of Results When Ashing Is Done at  $480^{\circ}$  in Silica*—The factors for converting cc of 0.04 or 0.02 N sodium hydroxide into mg or milli-

equivalents of potassium, used when the ashing is done in platinum, are multiplied by 1.044, the mean correction factor from Table III. The numerical constants in the formulae are therefore changed as follows:

Ashing in platinum

0.1657, 16.57

4.24

82.9

21.2

Ashing in silica

0.1730, 17.30

4.43

86.5

22.1

The solubility corrections, 0.009, 0.23, etc., are not changed.

TABLE I  
*Analysis of Standard Potassium Solutions*

Potassium present ( <i>k</i> )	0.04 N NaOH required ( <i>T</i> - <i>B</i> )	Potassium found* ( <i>f</i> )	Per cent deviation from theoretical $\frac{100 [(f) - (k)]}{(k)}$
mg	cc	mg	per cent
0.2620	1.539	0.2640	+0.70
0.2620	1.552	0.2662	+1.60
0.2620	1.538	0.2638	+0.69
0.5240	3.126	0.5269	+0.55
0.5240	3.089	0.5209	-0.59
0.5240	3.128	0.5271	+0.59
1.048	6.246	1.044	-0.38
1.048	6.215	1.039	-0.86
1.048	6.291	1.052	+0.38

\* Calculated by the formula,  $\text{mg of K} = 0.1657 (T - B) + 0.0090$

#### EXPERIMENTAL

##### *Analyses of Standard Potassium Chloride Solutions*

Standard solutions of recrystallized KCl were analyzed by the procedure given for 1 cc of serum or for urine. Ashing was in platinum. To cover the range of both serum and urine analyses, 50 mg of PTA were used for precipitation. The results are given in Table I.

##### *Tests of Ashing Conditions*

1. Possible errors due to volatilization of potassium sulfate were studied by heating samples of 260 to 580 mg of anhydrous  $\text{K}_2\text{SO}_4$  in a muffle. At  $480^\circ$  no weight loss occurred even when the heating was extended for 15 hours.

2 When crucibles or tubes of porcelain, glass, or silica were used for ashing, the results for potassium were low. The materials ranked in ascending order of the magnitude of error as follows: silica, Pyrex glass, porcelain. With unetched silica one may use a correction and obtain results usually within 5 per cent of correct values. When silica tubes or crucibles become etched, the potassium losses increase. Pyrex glass and porcelain caused errors that were too great to permit use of these materials. The errors appear to be due to fusion of some of the ash-potassium with the material of the container. The results are low, except that sometimes with porcelain some potassium seems to fuse out of the material and make results too high.

In Tables II and III are given the results of multiple analyses of three sera, in which part of the samples were ashed in platinum, part in silica. The values for the correction factor for loss in silica given in Table III are the values by which the coefficient 16.57, in the formula used for calculating serum potassium, must be multiplied when the formula is applied to samples ashed in silica to make the mean serum potassium, obtained after ashing in silica, equal that obtained when the ashing was in platinum. The average correction factor is 1.044.

*Comparison of Serum Potassium Analyses by Present Method with Analyses by Methods of Shohl and Bennett (14) and of Fiske and Litarczek (19)*

The results of the comparative analyses are given in Table IV. In all analyses the material was ashed in platinum crucibles and redissolved in 0.1 N HCl as described in this paper. For the Shohl-Bennett and Fiske-Litarczek methods the HCl solution thus obtained was concentrated to dryness and the methods of these authors were applied to the residues. In applying the Shohl-Bennett method we utilized the modification, introduced by Hald (20) and used by Consolazio and Talbott (18), of precipitating the  $K_2PtCl_6$  in alcohol which was presaturated with this substance, in order to insure complete precipitation. We employed Consolazio and Talbott's procedure of redissolving the ash residue in a drop of 1 N HCl and 0.3 cc of chloroplatinic acid solution, and then of adding 5 cc of alcohol previously saturated with  $K_2PtCl_6$ .

It was our experience that the end-point of the phosphotungstate titration was sharper and easier to distinguish than the end-point of the platinum chloride titration. In the latter the transition of the orange color of the  $K_2PtI_6$  to the yellow of  $K_2PtI_4$  is so gradual that some experience by the observer is necessary before he can with certainty stop at a uniform point.



TABLE II

*Comparison of Serum Analyses with Ashing in Platinum Crucibles Versus Ashing in Silica Crucibles or Silica Centrifuge Tubes*

Serum No	Furnace conditions for ashing		0.04 N NaOH required to titrate potassium phosphotungstate from 1 cc serum after ashing in		
	Temperature	Duration*	Pt crucibles	Silica crucibles	Silica centrifuge tubes
I	C 480	hrs 14	cc 1 124 1 121 1 150 1 139 1 134 1 137 1 154 1 146	cc 1 132 1 081 1 073 1 158 1 028	cc
Mean			1 138	1 095	
II	480	11	0 985 0 962	0 945 0 952 0 942	
	480	12	0 982 0 987	0 963 0 891 0 935 0 901	
	480	15	0 977 0 957 0 966	0 881 0 953 0 925 0 907	
	515†	16	0 961†	0 811† 0 825† 0 905†	
Mean			0 973	0 927	
III	480	14	0 901 0 875 0 908		0 872 0 843 0 840 0 803 0 886 0 887 0 874 0 884
Mean			0 895		0 861

\* From beginning of heating, at room temperature, to the time the crucible was removed from the hot furnace

† The data from ashing at 515° are included to show the apparent effect of higher temperature in increasing the loss in silica crucibles. These data are not included in calculating the mean or the standard deviation (Table III)

*Recovery of Potassium Added to Serum*

By the methods for 1 and 0.2 cc of plasma outlined in this paper analyses were carried out on sera to which known amounts of potassium from standard solutions of recrystallized KCl or K<sub>2</sub>SO<sub>4</sub> had been added. The results are summarized in Table V.

The potassium originally present in the sera had previously been ascertained by repeated analyses with the 1 cc method.

TABLE III

*Comparison of Serum Analyses with Ashing in Platinum Crucibles Versus Ashing in Silica Crucibles or Silica Centrifuge Tubes*

The calculations are from the data of Table II

	Serum I	Serum II	Serum III
$\bar{x}$ (Pt) = mean titration, after ashing in Pt, cc 0.04 N NaOH	1.138	0.973	0.895
No. of analyses with ashing in Pt	8	7	3
$\bar{x}$ (SiO <sub>2</sub> ) = mean titration, after ashing in silica, cc 0.04 N NaOH	1.095	0.927	0.861
No. of analyses with ashing in silica	5	11	8
Ratio, $\bar{x}$ (SiO <sub>2</sub> ) / $\bar{x}$ (Pt)	0.962	0.953	0.962
Mg K per 100 cc serum calculated from $\bar{x}$ (Pt)	19.76	17.02	15.73
Factor to correct for loss in silica	1.039	1.049	1.039
Mean factor from 24 analyses in silica to correct for loss in silica, 1.044			
Standard percentage deviation from mean	±1.2	±1.2	
Ashed in platinum, %	±2.9	±3.0	
" " silica crucibles, %			±2.9
" " " tubes, %			
$\sqrt{\frac{\sum \left( \frac{100(\bar{x} - x)}{\bar{x}} \right)^2}{N - 1}}$			
$\bar{x}$ = mean titration			
$x$ = individual titration			
$N$ = number of titrations			

*Analyses of Whole Blood*

Samples of 1 cc of whole blood were ashed and the ash solutions diluted to 10 cc with 0.1 N HCl as described above for whole blood analyses. Aliquots of 2 cc were analyzed by the method described in this paper and by Consolazio and Talbott's (18) modification of the method of Shohl and Bennett (14).

In order to find whether large amounts of iron would interfere with the phosphotungstate method, 2.04 mg of Fe in a 4 per cent solution of FeCl<sub>3</sub> · 6H<sub>2</sub>O were added to each 1 cc sample of blood in the last two

analyses, by the phosphotungstate method The results are given in Table VI

TABLE IV  
Comparison of Results with Serum and Plasma by Different Methods

Material	Authors' method				Shohl and Bennett's methods			Fiske and Litarczek (19) titration, 4 cc samples
	1 cc samples		0.2 cc samples		Titration (Consolazio and Talbott's modification) 1 cc samples	Colorimetric 1 cc samples		
	0.04 N NaOH	K per liter	0.02 N NaOH	K per liter				
							K per liter	
	cc	meq	cc	meq	meq	meq	meq	
Serum 1	1 150	5 10	0 438*	5 09	5 00	4 93		
	1 139	5 06	0 436	5 07	5 21	5 06		
	1 134	5 03	0 444	5 16				
	1 137	5 05	0 441	5 12				
	1 154	5 12	0 435	5 06				
	1 146	5 09	0 435	5 06				
	0 977	4 40	0 357	4 23	4 24		4 35	
	0 957	4 28	0 377	4 44	4 22		4 20	
	0 961	4 30	0 356	4 22			4 41	
	0 982	4 39	0 349†	4 32			4 44	
" 2	0 987	4 41	0 342†	4 24			4 48	
	0 985	4 41						
	0 962	4 31						
	0 966	4 33						
	0 992	4 43	0 392	4 60	4 53			
	1 028	4 59	0 389	4 57	4 56			
	1 247	5 52	0 486	5 60	5 56			
	1 091	4 85	0 420	4 89				
	0 832	3 76			3 91			
	1 016	4 53			4 24			
Plasma 1	0 965	4 32	0 361	4 28	4 47			
" 2	0 754	3 43	0 270	3 31	3 51			
" 3	1 155	5 13	0 432	5 03	5 00			
" 4	1 011	4 51	0 375	4 42				
" 5	1 634	7 15			7 20			
" 6	1 061	4 73			4 87			
" 7	0 763	3 46			3 27			

\* This series was originally titrated with 0.01 N NaOH

† These samples were analyzed in silica centrifuge tubes (see p 755)

### Urine Analyses

Comparison of results by the present method with results by Consolazio and Talbott's modification of Shohl and Bennett's method is given in Table VII

TABLE V  
Recovery of Potassium Added to Serum

Sample	K originally present in serum	K added	Titration (T - B)	K found (f)	K calculated (c)	Error $\frac{100 [(f) - (c)]}{(c)}$
cc	γ	γ	cc 0.04 N NaOH	γ	γ	per cent
1 0	198 4	200 0	2 353	398 9	398 4	+0 13
1 0		200 0	2 357	399 3		+0 23
1 0		200 0	2 340	396 8		-0 40
1 0		400 0	3 590	603 8	598 4	+0 90
1 0		400 0	3 573	601 0		+0 43
1 0		400 0	3 561	598 9		+0 08
1 0	170 1	97 75	1 545	265 0	267 85	-1 06
1 0		97 75	1 571	269 2		+0 50
1 0		97 75	1 555	266 7		-0 43
1 0		195 5	2 178	369 8	365 0	+1 15
1 0		195 5	2 182	370 5		+1 34
1 0		195 5	2 196	372 8		+1 97
Mean error						0 72
0 2	39 7	0	cc 0.02 N NaOH 0 435	39 6	39 7	-0 25
0 2		0	0 444	40 3		+1 51
0 2		0	0 438	39 8		+0 25
0 2		0	0 441	40 1		+1 01
0 2	39 7	40 0	0 936	81 1	79 7	+1 76
0 2		40 0	0 949	82 1		+3 01
Mean error						1 30

TABLE VI

Comparison of Results with 0.2 Cc Sample of Whole Blood by Different Methods

Modified Shohl Bennett method	Authors method			
K per liter	Iron added	0.04 N NaOH	K per sample	K per liter
mg	mg	cc	mg	mg
48 81		2 183	0 3708	47 42
48 58		2 168	0 3681	47 08
49 39	2 04	2 179	0 3700	47 31
48 70	2 04	2 198	0 3730	47 69

## SUMMARY

Potassium in the redissolved ash of serum, blood, urine, tissues, or feces is precipitated as the salt,  $K_5H(PO_4 \cdot 12WO_3) \cdot 6H_2O$ , of phospho-12 tungstic acid. The precipitate is washed with water by centrifugation, and is titrated with sodium hydroxide to form  $Na_2WO_4$  and  $Na_2HPO_4$ , 9.4 equivalents of NaOH being used for each equivalent of potassium.

The high alkali equivalent adapts the procedure to microdeterminations, from 1 to 0.2 cc of serum serves for an analysis.

TABLE VII  
Comparison of Results with Urine by Different Methods\*

Urine No	Consolazio and Talbott's modification of Shohl and Benedict's titrimetric method		Authors' method		
	Size of sample analyzed	K per liter	Size of sample analyzed	0.04 N NaOH	K per liter
	cc	meq	cc	cc	meq
1	0.2	51.6	0.2	2.446	53.0
		51.2		2.427	52.6
		54.2		2.410	52.2
		52.6		2.471	53.5
		52.3		2.261†	51.3†
2	0.2	53.5	0.2	2.507	54.2
3	0.2	45.7	0.2	2.137	46.4
4	0.2	42.6	0.2	2.048	44.5
5	1.0	5.5	1.0	1.433	6.3
6	0.2	57.60	0.2	2.559	55.4
7	0.2	106.0	0.2	4.920	105.4

\* The authors are indebted to Dr. William Beckman and Dr. Kendall Emerson, Jr., for analysis of an additional urine by the Fiske and Litarczek method, which gave 15.04 milliequivalents of K per liter compared with 15.35 by the authors' method.

† Determination carried out in the silica tube with ashing at 480°. The constant used in the calculation was therefore 4.43 instead of 4.24.

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# GRAVIMETRIC DETERMINATION OF POTASSIUM AS PHOSPHO-12-TUNGSTATE

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In the preceding paper (1) phospho-12-tungstic acid (PTA) was introduced for the quantitative precipitation of potassium. The potassium phosphotungstate (hereafter designated as KPT) was treated with an excess of standard alkali, and the split-products were titrated to an endpoint at pH 9.

In this paper a procedure is described in which the precipitate is dried at 97–100° and weighed as  $K_6H(PO_4 \cdot 12WO_3)_2 \cdot 6H_2O$ . Washing, drying, and weighing are carried out without transfer in a centrifuge tube. The 0.2 mg of potassium in 1 cc of average serum yields a precipitate of 6 mg, which is weighed to 0.01 mg on a semimicro balance.

The gravimetric technique, compared with the titrimetric, has the advantage of requiring fewer reagents, also, with a modern semimicro balance, the weighings can be done more rapidly than the titrations, as many as sixteen weighings can be done per hour. On the other hand the gravimetric method requires a minimum sample of 1 cc of serum, while the titration permits a minimum of 0.2 cc of serum.

The present procedure is described for amounts of potassium between 0.2 and 1.0 mg, but it appears from results in Table I that the method can easily be adapted to macroanalyses.

## Apparatus

*Platinum crucibles, electric muffle, 15 cc Pyrex centrifuge tubes marked at 0.2 and 0.5 cc, suction capillary, and Pyrex glass rods, all as described for the titration method (1).*

In addition the following are required:

*A semimicro balance<sup>1</sup> sensitive to 0.03 mg, or preferably 0.01 mg. It is convenient to have the supporting rods of the balance pans provided with hooks across which the 15 cc test-tubes can be laid for weighing.*

*A metal test-tube holder.*

*A pair of small crucible tongs with jaws lined with chamois skin, for placing test-tubes on the balance.*

*A drying oven with temperature set at 97–100°.*

\* Rockefeller Foundation Fellow in Biochemistry.

<sup>1</sup> We have used a Seoderer-Kohlbusch semimicro balance which carries as standard equipment on each side a pair of hooks attached to the pan supports.



*Reagents*

The phospho-12-tungstic acid, 4 per cent solution of PTA, approximately 4 N sulfuric acid, and 0.1 N hydrochloric acid, described in the previous paper (1)

## PROCEDURE FOR PLASMA OR SERUM

The analysis is carried out as described for a 1 cc sample in the preceding paper (1), through the point at which the precipitate is washed and dried in the centrifuge tube. Then the precipitate is weighed, instead of being redissolved and titrated. Special points are to be observed in the following details.

*Ashing of Sample*—At least 1 cc, preferably 2, 3, or 4 cc of serum or plasma are used for the gravimetric analysis. Per cc of plasma 0.5 cc of approximately 4 N sulfuric acid is added for the ashing.

*Precipitation of Potassium Phosphotungstate*—Of the 4 per cent PTA solution 0.5 cc is added per cc of plasma in the sample.

*Weighing the Empty Centrifuge Tubes*—The centrifuge tubes, because they are weighed to 0.01 mg, must be scrupulously clean inside and out. To prepare them they are washed with soap and hot water, rinsed with water, then completely immersed in chromic acid cleaning mixture for at least 30 minutes. During intervals when the tubes are not in use, it is good practice to keep them immersed in the cleaning mixture. Shortly before the tubes are used, the chromic acid is removed by three rinsings in tap water, followed by several with distilled water.<sup>2</sup>

The tubes are then drained, placed in a Pyrex beaker somewhat taller than the tubes, and dried in an oven at 97–100°. It is convenient to dry eight to twelve tubes in one beaker. After drying, until weighing is completed, the tubes are not touched by hand, but are handled with the tongs protected by chamois skin, or with a metal test-tube holder.

The dried tubes are cooled to room temperature, and, unless weighed at once, are placed, still in the beaker, in a desiccator. Before weighing is begun, the tubes and beaker are removed from the desiccator and let stand in the open air near the balance 20 minutes.

After the 20 minute equilibration the beaker is covered with an inverted Petri dish to protect the tubes from dust.

In order to compensate for the effects of changes in atmospheric conditions during the interval between the weighings of the empty tubes and

<sup>2</sup> The importance of the cleaning technique used for the centrifuge tubes is illustrated by the finding that the mean difference between duplicate successive weighings of the same tubes, with washing and drying preceding each weighing, was 0.02 mg when the tubes were cleaned by complete immersion in chromic acid, but was increased to  $\pm 0.09$  mg when only the inside of each tube was cleaned with chromic acid. There were twenty-five duplicate weighings in each of the two series.

of the tubes plus KPT, an empty tube, the lightest of the set, is placed on the right side of the balance and is used as counterpoise in weighing the other empty tubes. The latter in turn are removed with the chamois skin-protected pincers from the beaker, placed on the left side of the balance, weighed against the counterpoise, and returned to the beaker, which is kept covered except during the transfers. The weight recorded for each tube is the amount by which it exceeds the weight of the counterpoise tube.

*Transfer and Washing of Precipitate*—The precipitates are transferred to the weighed centrifuge tubes and washed, as previously described (1). Care is taken to stir up the precipitate thoroughly during the transfer into the weighed centrifuge tubes, and in the subsequent washings, in order to dissolve the last traces of calcium sulfate present.<sup>3</sup>

The outside walls of the centrifuge tubes must remain clean, to avoid errors in weight. Test-tube racks, metal containers for the tubes in the centrifuge, and the rubber pieces on which the tubes rest during centrifugation are therefore kept immaculate.

After the last washing of a precipitate the supernatant wash water is removed to the 0.2 cc mark. The outside walls of the centrifuge tubes are wiped with a clean towel and are rinsed with distilled water.

*Drying and Weighing the Tubes with Washed Precipitates*—The tubes containing KPT are placed in a beaker, dried, and weighed, as previously described for the empty tubes, with the same empty tube as counterpoise. The only difference in the handling of the tubes with KPT precipitates is that longer heating at 97–100° is required. To get the KPT to the composition  $K_6H(PO_4)_{12}WO_3)_2 \cdot 6H_2O$  from 2 to 4 hours at 97–100° are required. It does no harm to dry overnight at 97–100°. After drying, until the tubes have been weighed they are handled only with tongs or the metal test-tube holder.

*Blank Determination*—Two or more blank determinations, to correct for insoluble material formed from the reagents, are run with water in place of serum. The blank should be redetermined if the reagents have not been recently used.

*Calculation*—The potassium obtained in the sample analyzed is calculated as

$$\text{Mg K in sample} = 0.0332 (W - B)$$

$$\text{" " per 100 cc plasma} = \frac{3.32 (W - B)}{V}$$

$$\text{Milliequivalents K per liter plasma} = \frac{0.849 (W - B)}{V}$$

<sup>3</sup> For the titrimetric method (1) complete removal of the calcium sulfate is not essential, but for the gravimetric it is imperative. The solubility of calcium sulfate is such that the water used in washing the KPT could dissolve about 15 to 16 mg of calcium.

$W$  = mg of precipitate obtained from the sample,  $B$  = mg of precipitate obtained from the blank,  $V$  = cc of serum used for analysis

*Example*—A 3 cc plasma sample was analyzed. The empty sample tube outweighed the counterpoise tube by 175.66 mg. The sample tube containing the washed and dried precipitate outweighed the counterpoise tube by 192.02 mg. Hence the weight of the precipitate =  $192.02 - 175.66 = 16.36$  mg. In a blank determination the weight of the precipitate was 0.12 mg. Hence  $W - B = 16.36 - 0.12 = 16.24$  mg, and milliequivalents of K per liter of plasma =  $\frac{0.849 \times 16.24}{3} = 4.60$

*Titrimetric Check*—The results of gravimetric analysis can be checked, after the precipitate has been weighed, by titrating it as described in the preceding paper (1). Such a check may save an analysis in case of an accident affecting the weight of the centrifuge tube.

#### EXPERIMENTAL

##### *Determination of the Potassium Equivalent of the KPT Precipitate*

The gravimetric factor, K KPT, was determined by measurements of the weights of the KPT precipitates formed from known weights of potassium. Standard potassium solutions were made from recrystallized KCl and  $K_2SO_4$ . For conditions of the usual microanalysis, standard solutions containing 0.2 to 1.0 mg of potassium were pipetted into platinum crucibles and evaporated to dryness on the steam bath. The residues were redissolved in 0.5 cc portions of 0.1 N HCl, and the KPT was precipitated, transferred to weighed centrifuge tubes, and washed, dried, and weighed as described above.

To cover also the conditions of macroanalyses, solutions containing 8 mg of potassium were measured into platinum dishes of about 100 cc capacity and evaporated to dryness. The residue from each solution was redissolved in 10 cc of 0.1 N HCl, and 0.8 gm of PTA (10 cc of 8 per cent solution) was added. Each solution was evaporated to dryness, and the KPT precipitate in the residue was transferred to a 240 cc centrifuge bottle. The KPT was washed successively with 240, 140, and 140 cc of water, the total of 520 cc being 20 times the volume used in the microanalyses. The KPT residue was finally transferred back to the platinum dish with about 30 cc of water, and was dried at  $100^\circ$  and weighed in the dish.

The results obtained under both micro and macro conditions are given in Table I.

In the fourth column of Table I the factor, K KPT, is calculated as the ratio of mg of potassium in the sample to the observed mg of KPT precipitate. The ratio, averaging 0.0332, appears to be independent of variations in sample size within the range covered by the method.

In the last two columns of Table I the factors are recalculated with correction for the solubility of the KPT. From the data in Table II, the amount of KPT that is soluble in the 26 cc of wash water used in the micro technique is estimated to be  $0.26 \times 1.16 = 0.30$  mg and the amount soluble in the 520 cc used for the macroanalysis is estimated at 6.0 mg. The mean corrected factor in the last column of Table I is nearer to the theoretical value, 0.03224, for the ratio  $K_2K_5H(PO_4)_{12}WO_3)_2 \cdot 6H_2O$ , than is the mean uncorrected factor in the fourth column.

However, it appears from the results that more constant values for the gravimetric factor with varying amounts of potassium are obtained when no correction for solubility is made. Hence the factor 0.0332 has been adopted to calculate potassium from observed weights of KPT obtained in either

TABLE I  
*Determination of Gravimetric Factor*

Potassium present K	No. of determinations	Average weight of ppt. W	Gravimetric factor uncorrected for solubility $F = \frac{K}{W}$	Volume of wash water used	Weight of potassium phosphotungstate soluble in wash water used S	Weight of ppt. corrected for solubility $W + S$	Gravimetric factor corrected for solubility $F = \frac{K}{W + S}$
mg		mg		cc	mg	mg	
0.200	9	6.01	0.0333	26	0.30	6.31	0.0317
0.400	11	12.08	331	26	0.30	12.38	323
0.600	6	17.96	334	26	0.30	18.26	328
1.000	5	30.21	331	26	0.30	30.51	327
8.00	4	241.6	331	520	6.0	247.6	323
Mean			0.0332				0.0324

\* Corrected for blank

macro or micro gravimetric analyses, 1 mg of potassium yielding  $1/0.0332 = 30.1$  mg of precipitate.

#### *Solubility of Potassium Phospho-12-tungstate*

Potassium phosphotungstate was prepared by precipitating the K of 380 mg of KCl with 5 gm of PTA and washing the precipitate three times in the centrifuge with 40 cc portions of water. With each portion of wash water the centrifuge tube was stoppered and shaken thoroughly before centrifuging. The washed KPT was dried at room temperature in a desiccator over calcium chloride. For solubility determinations three 1 gm portions of the KPT were placed in 250 cc centrifuge bottles with 230 cc portions of water. The bottles were stoppered and shaken for 3 hours, and then centrifuged at 3000 R P M for 45 minutes. The supernatant

fluid was drawn, and, although water-clear, was filtered through a hardened filter paper. Of each filtrate a 200 cc portion was evaporated on the steam bath, first in a 400 cc Pyrex beaker, then in a weighed 50 cc silica beaker, in which the residue was finally dried at 100°, and weighed (Determinations 1, 2, and 3, Table II)

The KPT left in the bottles was dried at 100° for 2.5 hours, and about 250 mg of each portion were removed for analysis. To the remaining 0.75 gm 230 cc of water were added and each mixture was again shaken for 3 hours and centrifuged. The supernatant solution was centrifuged and filtered, and 200 cc portions were concentrated, and the residues were dried and weighed as before (Determinations 4 and 5, Table II)

It is evident from Table II that the preparation used for the first three determinations was as pure as it could be made by washing, since the

TABLE II  
*Solubility of Potassium Phosphotungstate*

Material	Determination No	Temperature	Solubility per 100 cc water
		C	mg
Preparation A washed 3 times	1	25.5	1.3
	2	25.5	1.1
	3	25.5	1.3
Preparation A washed 4 times	4	24.6	1.1
	5	24.6	1.0
Mean			1.16

additional washing by a great volume of water did not change the solubility. The solubility of 1.16 mg of KPT per 100 cc is equivalent to 0.038 mg of potassium per 100 cc. In the 26 cc of wash water used in the routine micro potassium determination, both titrimetric (1) and gravimetric, 0.010 mg of potassium as KPT would dissolve. This corresponds closely to the correction of 0.009 mg found in working out the titration method (1), and makes it appear that the wash water becomes practically saturated.

The potassium-12-phosphotungstate preparations obtained by the described technique were practically insoluble in absolute alcohol, amyl alcohol, and ether, and could not be extracted from an acidified water suspension by ether-amyl alcohol mixture. In excess cold dilute NaOH solution the KPT dissolves, presumably as a result of decomposition into phosphate and tungstate.

#### *Tungstic Acid Content of Potassium Phosphotungstate*

The method of Barber (2) with tannic acid as precipitating agent was used in analyses of the KPT preparations that were employed for solubility

determinations. The precipitates of the tungstic acid-tannic acid complex were washed by centrifugation in 250 cc centrifuge bottles. The washed precipitates were transferred to ash-free filter paper, dried at 100° in weighed platinum dishes, and ignited overnight in a muffle at 550°. The  $\text{WO}_3$  found was 91.6 and 92.2 per cent of the KPT, calculated for  $\text{K}_3\text{H}(\text{PO}_4)_2 \cdot 12\text{WO}_3 \cdot 6\text{H}_2\text{O}$ , 91.8 per cent.

### *Weight Loss of Potassium Phosphotungstate on Ignition*

The water content of three KPT preparations previously dried at 100° was determined by ignition. Preparation A was the one used for solubility

TABLE III  
*Weight Loss of Potassium Phosphotungstate on Ignition*

Material	Loss of weight on ignition	
	Found	Calculated for $\text{H}_2\text{O}$ of $\text{K}_3\text{H}(\text{PO}_4)_2 \cdot 12\text{WO}_3 \cdot 6\text{H}_2\text{O}$ *
	<i>per cent</i>	<i>per cent</i>
Preparation A washed 5 times	1.73	1.78
	1.87	
	1.90	
Preparation B	1.65	
	1.70	
	1.68	
" C	1.64	
Mean	1.74	

\* Calculated on the assumption that the loss on ignition is that of the assumed 6 molecules of water of crystallization.

determination. Preparations B and C were prepared under the same conditions detailed for preparing KPT for weighing in plasma analysis, except that larger amounts were taken.

Preparations A and B (Table III) were ignited by the technique of Toennies and Elliott (3). Preparation C was ignited overnight in a muffle at 500° in platinum crucibles. Additional ignition with the technique of Toennies and Elliott did not produce any further weight loss.

When the ignition is applied to PTA or its salts with amino acids, the phosphoric acid is converted into  $\text{HPO}_3$  (3), so that 1 molecule of water can be deducted for this change and the rest assigned to water of crystallization. In ignition of KPT, however, it is uncertain what changes occur to the  $\text{PO}_4$ .

### *Amount of Sodium Hydroxide Required to Titrate Potassium Phosphotungstate*

Samples of 90 to 180 mg of potassium phosphotungstate, Preparation A, were heated with an excess of 0.04 N NaOH and titrated as described in the

preceding paper for 1 cc samples of plasma. The results are given in Table IV.

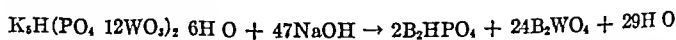
It appears from Table IV that with increasing washing of the precipitate the amount of NaOH required for the titration of 1 mg of substance diminishes, slightly but definitely, the difference between the thrice washed and five times washed precipitates exceeding the probable error. For the four times washed Preparation A, the required alkali approximates most

TABLE IV  
*Titration of Potassium Phosphotungstate*

Material	0.04 N NaOH required for titration of 1 mg of preparation	Equivalent weight* by titration to pH 9
	cc	mg ppt per cc 1 N NaOH
Preparation A washed 3 times	0.1936	
	0.1967	
	0.1952	128.1
Preparation A washed 4 times	0.1928	
	0.1941	
	0.1935	129.2
Preparation A washed 5 times	0.1925	
	0.1911	
	0.1915	
	0.1920	
	0.1918	
	0.1918	130.4

\* If the potassium phosphotungstate molecule is  $K_5H(PO_4 12WO_3)_2 \cdot 6H_2O$ , with a molecular weight of 6063, 47 equivalents of NaOH should be required to titrate 1 mole to  $B_2WO_4$  and  $B_2HPO_4$ , and the equivalent weight should be  $6063/47 = 129.0$

closely that calculated for the composition  $K_5H(PO_4 12WO_3)_2 \cdot 6H_2O$ , according to the equation,



which indicates an equivalent weight of 129.0 (B on the right side of the equation represents either K or Na)

#### *Results with Standard Potassium Solutions*

Representative analyses of standard potassium solutions are shown in Table V. They were performed by the technique described in this paper.

TABLE V  
Analysis of Standard Potassium Solutions

Potassium present K	Weight of ppt		Deviation from calculated
	Found (H - B)	Calculated as $\frac{K}{0.0332}$	
mg 0.200	mg 6.12 6.09 5.81 5.84 6.17 5.91	mg 6.02	per cent +1.5 +1.1 -3.5 -3.0 +2.4 -1.9
Mean	5.99		
Standard deviation, mg	±0.15		
“ “ %	±2.6		
0.400	11.95 12.36 12.15 12.08 11.82 12.02	12.05	-0.8 +2.6 +0.8 +0.3 -1.9 -0.3
Mean	12.06		
Standard deviation, mg	±0.18		
“ “ %	±1.5		
0.600	17.94 18.22 18.10 17.78 17.84 17.90	18.07	-0.7 +0.8 +0.2 -1.7 -1.3 -1.0
Mean	17.96		
Standard deviation, mg	±0.17		
“ “ %	±0.9		
1.000	29.90 30.13 30.46 30.35 30.21	30.12	-0.7 ±0.0 +1.1 +0.7 +0.3
Mean	30.21		
Standard deviation, mg	±0.21		
“ “ %	±0.7		
1.500	45.37 46.13	45.18	+0.3 +2.1
2.000	61.69 60.45	60.24	+2.5 +0.4
3.000	90.45 90.64	90.36	+1.0 +0.3

Standard deviation =  $\sqrt{\sum d^2 / (n - 1)}$ , where  $d$  = deviation from the mean,  $n$  = number of determinations



for 1 cc serum samples. For amounts of potassium above 1.0 mg correspondingly more PTA reagent was used, but the same volume of wash water, 26 cc, was used in all analyses.

Over the range from 0.2 to 1.0 mg of potassium per sample the standard deviation, in mg of precipitate, is approximately constant, so that the percentage error is in inverse proportion to the size of the sample. It appears from comparison with Table II of the preceding paper (1) that for accuracy equal to that of the titration method (1) about 2.5 times as large a sample is required for the present gravimetric analysis. While the 0.2 mg of K in 1 cc of serum suffices for a gravimetric analysis with a standard deviation of  $\pm 2.6$  per cent, if 3 cc of serum are used the standard deviation is cut to  $\pm 0.9$  per cent.

If the sample much exceeds 1 mg of potassium, it appears that the wash technique, with 26 cc of wash water, may not suffice to remove quite all of the excess PTA, the results in Table V with 1.5 to 3.0 mg of potassium are all from 0.3 to 2.5 per cent above theoretical. On the other hand, in Table II it has been shown that samples with as much as 8 mg of K give a theoretical yield of KPT when the volume of wash water is increased in proportion to the amount of potassium. The present method is described for amounts of potassium between 0.2 and 1.0 mg, but it appears that if 12 instead of 7 cc of water were used for the second and third washings, samples up to 3 mg should give exact results.

*Comparison with Results of Blood Analyses by Titration Methods of Fiske and Litarczek and the Authors*

Results obtained with human serum, human plasma, dog plasma, horse serum, and horse plasma with different methods are compared in Tables VI and VII. For comparison the authors' titrimetric procedure (1) and the titration method of Fiske and Litarczek (4), in which double precipitation and filtration and titration of the potassium acid tartrate are employed, were used. The results are given in Tables VI and VII.

*Influence of Other Inorganic Blood Constituents*

Samples containing 0.010 milliequivalent of potassium (the amount in about 2 cc of serum), and samples to which sodium, or sodium and calcium, or sodium, calcium, and magnesium had been added in the approximate proportions found in human serum were analyzed by the gravimetric method. Per 1 equivalent of  $K^+$ , up to 28 equivalents of  $Na^+$ , 1 equivalent of  $Ca^{++}$ , and 0.5 equivalent of  $Mg^{++}$  were added as chloride or nitrate. In no case did the weights of the washed KPT precipitates differ from the weight obtained from the sample containing only potassium.

In experiments in which the influence of anions was studied, similar results were obtained, and were confirmed by the titrimetric method (1). In these experiments platinum crucibles were charged with 40 mg each of PTA in 0.5 cc of solution. After evaporation to dryness, the usual procedure described for analysis of 1 cc portions of serum was followed. The

TABLE VI  
*Comparison of Titrimetric and Gravimetric Determinations of Serum Potassium As Phosphotungstate*

Material	Titrimetric*		Gravimetric		
	Volume of sample	K per liter	Volume of sample	Weight of ppt	K per liter
	cc	meq	cc	mg	meq
Horse serum 1	1	3.89	1	4.42	3.75
" " 2	1	4.01	1	4.85	4.12
" " 3	1	4.12	1	4.81	4.08
" " 4	1	4.12	1	4.78	4.06
			1	4.88	4.14
			1	4.83	4.10
			2	9.80	4.16
			2	9.83	4.17
" " 5	0.2	4.26 4.22	4	20.37	4.32
Human serum 1	1	4.22	1	5.04	4.28
" " 2	1	3.94	1	4.80	4.07
" " 3	1	4.08	1	4.78	4.06
" " 4	1	5.08	1	6.12	5.20
" " 5	1	4.32	2	9.84	4.18
" " 6	2	4.24	2	10.17	4.32
" " 7	2	4.27	2	10.23	4.34
" " 8	1	4.47	3	16.24	4.59
Human plasma 1	1	4.66	1	5.47	4.64
" " 2	1	3.75	1	4.29	3.64
Dog plasma	1	4.25	3	14.91	4.22

\* Van Slyke and Rieben (1)

following substances were added to different crucibles in the amounts indicated:  $\text{H}_2\text{PO}_4$ , 1 mg,  $\text{Na}_3\text{PO}_4 \cdot 12\text{H}_2\text{O}$ , 13 mg,  $\text{Na}_2\text{SO}_4$ , 1 mg,  $\text{Na}_4\text{P}_2\text{O}_7 \cdot 10\text{H}_2\text{O}$ , 20 mg,  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ , 8 mg. None of these additions produced any noticeable interference.

In the cases of urine and muscle analyses, in which ashing losses are

prevented by addition of anhydrous sodium sulfate, it was found that up to 0.200 milliequivalent of  $\text{Na}_2\text{SO}_4$  could be added per sample without perceptible variations in the results. When 0.400 milliequivalent was added, the results for potassium were about 2 per cent too low.

TABLE VII

*Comparison of Serum Analyses by Fiske-Latarczek Titrimetric Method with Analyses by Present Gravimetric Method*

Material	Fiske and Latarczek method (4)		Present gravimetric method		
	Volume of sample	K per liter	Volume of sample	Weight of ppt (W - B)	K per liter
	cc	mg	cc	mg	mg
Human serum	4	4.35	1	5.07	4.30
		4.20		4.96	4.21
		4.41			
		4.44			
		4.48			
Horse "	4	6.75	0.5	4.11	6.98
		6.89	1.0	8.13	6.90
				7.74	6.57
			2.0	15.57	6.61
			3.0	23.53	6.66
Human plasma	4	5.91	2.0	13.68	5.81
		6.34			
		5.91			
		5.87			

## SUMMARY

Potassium is precipitated with phospho-12-tungstic acid, and the precipitate is dried at  $100^\circ$ , and weighed as  $\text{K}_5\text{H}(\text{PO}_4 \cdot 12\text{WO}_3)_2 \cdot 6\text{H}_2\text{O}$ . Washing, drying, and weighing are done in the same centrifuge tube, without transfer.

The solubility of the precipitate in water at room temperature is 1.16 mg per 100 cc, equivalent to 0.038 mg of potassium per 100 cc.

The low solubility and high equivalent weight of the precipitate adapt it to gravimetric microanalysis, 1 mg of potassium yielding 30.1 mg of precipitate. 1 to 3 cc of serum provides a sample for the analysis, with weighing of the precipitate on a semimicro balance.

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## LETTERS TO THE EDITORS

### NOTE ON CHANGES IN HORSE SERUM ALBUMIN ON AGING

Sirs

In connection with a related investigation, we have observed that five times recrystallized horse serum albumin, prepared according to Adair and Robinson,<sup>1</sup> showed two electrophoretic components in sodium phosphate buffer at pH 7.4 after 46 months storage in the ice box as a sterile, aqueous isoelectric solution (Fig 1, a). A culture on blood agar

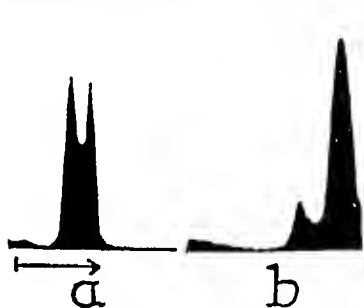


FIG 1

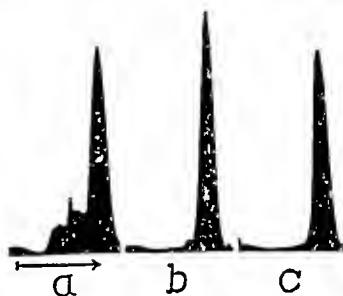


FIG 2

FIG 1 Electrophoretic pattern of five times crystallized horse serum albumin stored in the ice box for (a) 46 months, (b) 20 months

FIG 2 Electrophoretic pattern of fresh horse serum albumin after (a) initial crystallization, (b) first, and (c) second recrystallizations

plates indicated no bacterial or other contamination. The slower component, constituting about 57 per cent of the total protein, had a mobility of  $-3.9 \times 10^{-5}$  sq cm per second per volt, whereas the faster component had a mobility of  $-5.3 \times 10^{-5}$  sq cm per second per volt, which approximates that of freshly prepared crystalline albumin.

A fresh lot of albumin prepared as above was examined electrophoretically and found to be homogeneous after the second recrystallization (Fig 2, c). It is interesting to note, however, that a large amount of  $\alpha$ - and  $\beta$ -globulins was present in the initial crystals (Fig 2, a) and that recrystallizations resulted in substantial purification (Fig 2, b and c).

The two components of the 46 months-old albumin were electropho-

<sup>1</sup> Adair, G. S., and Robinson, M. E., *Biochem J.*, **24**, 993 (1930)

retically separated and found to have the same sedimentation constant,  $S_{20} = 4.4$  Svedberg units. Diffusion constants differed, however, being  $D_{20} = 6.3 \times 10^{-7}$  sq cm per second and  $D_{20} = 4.5 \times 10^{-7}$  sq cm per second for the faster and slower components, respectively. Assuming a partial specific volume of 0.75, the calculated molecular weights would be 68,000 and 95,000. Frictional ratios calculated from the above data are 1.24 for the fast and 1.56 for the slow fraction. If the molecules are assumed to be oblong ellipsoids, the corresponding axial ratios would be 5 and 10 respectively.

Another sample, having stood 20 months, contained only 17 per cent of the slow component (Fig 1, b).

Preliminary experiments indicate that a discernible slow component can be produced from originally homogeneous albumin within 2 months at room temperature and within a few hours at higher temperatures. Details of these experiments will be reported later.

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# THE EFFECT OF ADRENALECTOMY, ADRENAL CORTEX EXTRACT, AND ADRENAL CORTEX EXTRACT PLUS TESTOSTERONE PROPIONATE ON THE PHOSPHATASES OF RAT TISSUES\*

Sirs

Certain of the  $C_{11}$  oxygenated adrenal cortical hormones cause glyconeogenesis from protein<sup>1</sup> On the other hand, certain androgens stimulate protein anabolism<sup>2</sup> It seemed, therefore, that the simultaneous administration of these steroids would provide information concerning their mode of action in the body As one means to this end, the "alkaline" and "acid" phosphatases of the liver and kidney were studied It was assumed that the adrenal cortex influenced these enzymes to bring about its effect on carbohydrate metabolism<sup>3</sup>

Male white rats weighing 140 to 240 gm were used (see the table) The animals were given 1 per cent sodium chloride solution for drinking water The testosterone propionate<sup>4</sup> injections were begun on the 1st day after the operations, on the 4th day the food was removed, and on the 5th day the adrenal cortex extract<sup>5</sup> was injected<sup>6</sup> 1 to 2 hours after the last injection, the rats were anesthetized with sodium amytal The tissues were removed and homogenized<sup>7</sup>

The enzymes were determined as previously described<sup>7</sup> except that the "acid" phosphatase was determined at pH 5.4

The increase in "alkaline" phosphatase of the liver, as a result of adrenalectomy and especially injections of adrenal cortex extract, indicates that the effect of the adrenal cortex on carbohydrate metabolism is by way of the phosphorylated intermediates Apparently, the "acid" phosphatase of the liver is not involved in the above processes The situation in the kidney, however, is entirely different In this organ adrenalectomy has no effect on the "alkaline" phosphatase but decreases the "acid" phosphatase which is restored to, but not greater than, normal on the administration of adrenal cortex extract

\* This investigation was aided by a grant from the Josiah Macy, Jr., Foundation

<sup>1</sup> Long, C. N. H., Katzin, B., and Fry, E. G., *Endocrinology*, **26**, 309 (1940)

<sup>2</sup> Kochakian, C. D., *Am. J. Physiol.*, **142**, 315 (1944) Kenyon, A. T., Knowlton, K., and Sandiford, I., *Ann. Int. Med.*, **20**, 632 (1944)

<sup>3</sup> Cori, C. F., in A symposium on respiratory enzymes, Madison, 175 (1942)

<sup>4</sup> Ciba Pharmaceutical Products, Inc., provided the testosterone propionate (perandren)

<sup>5</sup> Dr. M. H. Kuizenga, The Upjohn Company, provided the adrenal cortex extract

<sup>6</sup> Reinicke, R. M., and Kendall, E. C., *Endocrinology*, **31**, 573 (1942)

<sup>7</sup> Kochakian, C. D., and Fox, R. P., *J. Biol. Chem.*, **153**, 669 (1944)

The inability of testosterone propionate to influence the effect of the adrenal cortex extract on the "alkaline" phosphatase of the liver or the 'acid' phosphatase of the kidney indicates that under the conditions of the experiment there is no competition by the two types (protein anabolic and protein catabolic) of hormones for the intermediary products

*Effect of Adrenalectomy, Adrenal Cortex Extract, and Adrenal Cortex Extract Plus Testosterone Propionate on Phosphatases of Rat Tissues*

Group	No of rats	Average body weight gm	Liver phosphatase				Kidney phosphatase			
			'Alkaline'		'Acid'		'Alkaline'		'Acid'	
			units per gm	per cent	units per gm	per cent	units per gm	per cent	units per gm	per cent
Normal	6	216	3.6		23.2		22.4		20.2	
Adrenalectomized	8	204	4.6	+27	21.7	-6	21.8	-3	15.4	-24
Adrenalectomized + C E *	8	151	16.8	+366	21.5	-7	24.1	-7	19.9	-2
Adrenalectomized + C E + T P †	8	163	16.7	+364	22.5	-3	33.0	+52	19.5	-3

\* 1 cc of adrenal cortex extract (aqueous, Upjohn) every hour for 8 hours on the 5th day after adrenalectomy

† 25 mg of testosterone propionate twice a day

The increase in "alkaline" phosphatase in the kidneys of the rats receiving testosterone propionate probably is due to the androgen *per se*. Similar results have been obtained in rats that have received only testosterone propionate (Kochakian, unpublished).

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## 2-KETO-D-GLUCONIC ACID IN THE POLYSACCHARIDE OF IRISH MOSS

Strs

Galactose has long been recognized as a constituent of the polysaccharide of Irish moss (*Chondrus crispus*) and a recent estimate<sup>1</sup> has placed this hexose as comprising 34 per cent of the total molecule. Furthermore the polysaccharide is an ethereal sulfate with a total ash content<sup>1</sup> of about 18.7 per cent. The remainder of the molecule is not known. We wish to report the isolation in considerable quantity of 2-ketogluconic acid as a constituent of this polysaccharide.

A purified sample of the polysaccharide was prepared by aqueous extraction of the algal fronds at 20°, precipitation with ethanol, and dialysis. It had an ash content of 18.4 per cent. This product was hydrolyzed with oxalic acid in the presence of potassium oxalate in an atmosphere of nitrogen. The alcohol-soluble material of the residue was treated with absolute acetone and anhydrous copper sulfate to form the acetone derivative. The distillate at a pressure of 1 cm. of Hg formed crystals which on further purification had a melting point of 95° and  $[\alpha]_D^{22} = -48.8^\circ$ . Elementary analysis showed C 52.99 and H 6.81 per cent. Constants for diisopropylidene-2-ketogluconic acid are given as m.p. 96–97°,  $[\alpha]_D^{22} = -49.4^\circ$ , C 52.72, H 6.60.<sup>2</sup> This compound was prepared synthetically and the mixed melting point with our product was the same as the original. The anhydride was prepared showing a melting point at 122° and  $[\alpha]_D^{22} = -28.9^\circ$  as against the recorded values<sup>2</sup> of 120° and  $-31.3^\circ$ . The physical constants of the methyl ester, m.p. 52° and  $[\alpha]_D^{22} = -43.9^\circ$ , also agree with the recorded values of Ohle and Wolter.<sup>2</sup>

After hydrolysis of the polysaccharide with hydrochloric acid in air (though not with oxalic acid in nitrogen) a crystalline compound was isolated which appeared to be the lactone of arabonic acid. This was established on the basis of the melting points of the phenylhydrazide and the tribenzoyl derivatives.

These findings suggest an explanation for previous claims of the occurrence of fructose and pentose in the structure of this polysaccharide. 2-Ketogluconic acid could yield arabinose on decarboxylation under suitable conditions, and we have found that it furnishes the color reactions of fructose.

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<sup>1</sup> Buchanan, J., Percival, E. E., and Percival, E. G. V., *J. Chem. Soc.*, 51 (1943).

<sup>2</sup> Ohle, W., and Wolter, R., *Ber. chem. Ges.*, 63, 843 (1930).





## THE FORMATION OF MALONIC ACID FROM OXALACETIC ACID BY PIG HEART PREPARATIONS\*

Sirs

We have found in pig heart an enzyme which catalyzes the oxidation of oxalacetic acid by molecular oxygen. Malonic acid has been identified as a product of the reaction.

Water extracts of acetone powders of several other tissues (pig liver, pigeon breast muscle, rabbit heart, rabbit brain, rat brain) cause an oxidative removal of this metabolite. Freshly ground pig heart tissue yields more active extracts than the acetone powder. Dialysis of the extracts results in inactivation, reactivation can be effected by  $\text{MnCl}_2$ . Boiling for 3 minutes also leads to inactivation. The activity can be concentrated by ammonium sulfate precipitation. The protein fraction obtained between 50 and 100 per cent saturation is active, whereas the more soluble fraction is completely inactive. The reaction can be followed manometrically by measuring oxygen consumption and carbon dioxide evolution by the usual Warburg techniques in acetate buffer, pH 5.0, or in glycerophosphate buffer of the same pH. Under these conditions some oxalacetic acid is always decarboxylated to pyruvate, but most of it disappears with the equivalent absorption of 0.5 mole of oxygen and the production of 1 mole of carbon dioxide, as shown in the figure (Pyruvic acid itself is oxidized by the enzyme. The rate is very slow at the pyruvate concentrations attained as a result of the decarboxylation).

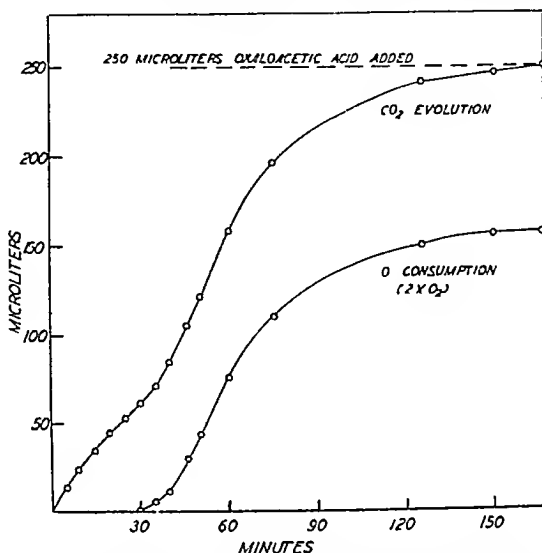
These stoichiometric relations suggest that the product might be malonic acid, formed according to the equation,  $\text{COOH-CH}_2\text{-CO-COOH} + \frac{1}{2}\text{O}_2 \rightarrow \text{COOH-CH}_2\text{-COOH} + \text{CO}_2$ . Experiments are in progress to determine the fate of malonic acid in the animal body.

An enzyme reaction mixture similar to the one described in the figure but containing 235 ml. of enzyme (prepared by extracting freshly ground pig heart with 3 volumes of water and dialyzing the extract) was incubated in air at 30° with 400 mg. of oxalacetic acid. After the oxygen consumption had ceased, the protein was precipitated with metaphosphoric acid. The filtrate was extracted with ether in the presence of bisulfite. A white crystalline acid (1.5 milliequivalents) was obtained from the extract and treated with *p*-nitrobenzyl bromide according to Reid<sup>1</sup>. After one re-

\* Aided in part by grants from the John and Mary R. Markle Foundation and from the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago.

<sup>1</sup> Reid, E. E., *J. Am. Chem. Soc.*, **39**, 131 (1919).

crystallization and careful washing, the product weighed 65 mg and melted at 86°, as did a sample of known di-*p*-nitrobenzyl malonate. Further recrystallizations did not change the melting point and there was no de-



Pig heart acetone powder extracted with 8 volumes of water and dialyzed 24 hours against M/40 phosphate buffer, pH 7.4. The vessels contained 0.7 ml of enzyme, 0.5 ml of 0.5 M glycerophosphate buffer, pH 5.0 (prepared from sodium  $\alpha, \beta$  glycerophosphate and nitric acid), 0.2 ml of 0.01 M  $\text{MnCl}_2$ , and water to make a final volume of 2.0 ml. At zero time, oxalacetic acid was tipped in from the side arm. No gas exchange occurred when no oxalacetic acid was added. The reaction was run in Warburg vessels in air at 30°. Oxalacetic acid added, 250 microliters,  $\text{CO}_2$  formed, 250 microliters, pyruvate formed, 118 microliters,  $\text{O}_2$  consumed, 78 microliters, pyruvate +  $2 \times \text{O}_2$ , 274 microliters.

pression of the mixed melting point. Analysis gave 54.36 per cent C, 3.82 per cent H, and 7.40 per cent N, no ash. (Theory, 54.55 per cent C, 3.77 per cent H, 7.48 per cent N.)

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# BIOLOGICAL VALUES AND TRUE DIGESTIBILITIES OF SOME FOOD PROTEINS DETERMINED ON HUMAN SUBJECTS

Sirs

In the course of an investigation<sup>1</sup> of the nutritive value of proteins in relation to the essential amino acids it was found necessary to determine the biological value of the proteins used in relation to the endogenous nitrogen excretion determined on a no protein period of 3 days just preceding the diet containing the test protein. This no protein diet supplied from 0.22 to 0.35 gm. of nitrogen as an average daily intake in different periods. Several different diet squads were concerned in this investigation.

On one squad consisting of seven subjects, five men and two women, the biological value of whole egg protein was found to be 97 in a scale of 100, with a range of 92 to 100. On the same squad the biological value of soy bean protein was 81 (range, 72 to 92). And on the same squad the biological value of beefsteak protein was 84 (range, 72 to 93).

On another squad of eleven subjects (all young men) ten of them (one made an error) gave the biological value for peanut protein as 83 (range, 67 to 95). On the same squad for "kitchen food"<sup>2</sup> yeast the eleven subjects gave a biological value of 87 (range, 76 to 96). And on the same squad, less one subject who was retired on account of illness, the biological value of cottonseed protein for nine of the ten subjects (one made an error) came out 91 (range, 82 to 103).

We believe these values will be of interest to all persons engaged in the evaluation of proteins for human consumption. It may be pointed out that in the experience of this laboratory an intake of protein above the level indicated by the endogenous excretion of nitrogen always gives a lower biological value than the ones here obtained. The reason for limiting the no protein period to 3 days is the difficulty human subjects experience in taking such a diet without injury to the power of digesting protein. This appears to be a reduction in the secretion of digestive fluids. A longer period than 3 days, therefore, requires a longer period of adjustment before full digestibility of the test protein is attained. The true digestibility, so called (that is, comparing the fecal excretion on the protein diet with the fecal excretion on the no protein diet), for these several proteins was as follows: egg protein 96, soy bean protein 89, beefsteak protein 97, peanut protein 93, "kitchen food" yeast protein 87, and cottonseed protein 78. Obviously, true digestibility does not determine biological value and, by the same token, nitrogen balances do not correctly indicate the true food

<sup>1</sup> Done under contract with the Office of Scientific Research and Development

<sup>2</sup> Supplied by Anheuser Busch, Inc., St. Louis, Missouri

value of a protein so convincingly as does the determination of biological value in the technical sense, biological value being defined as the percentage of the absorbed nitrogen which is retained for synthesis of body protein. We have confirmed the supremacy of whole egg protein as first reported by Mitchell and Carman.<sup>3</sup>

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